



Aalborg Universitet

**AALBORG UNIVERSITY**  
DENMARK

## **Optimization of the wet explosion pretreatment for increasing biogas and bioethanol yield of lignocellulosic biomass**

Biswas, Rajib

*Publication date:*  
2012

*Document Version*  
Early version, also known as pre-print

[Link to publication from Aalborg University](#)

*Citation for published version (APA):*  
Biswas, R. (2012). *Optimization of the wet explosion pretreatment for increasing biogas and bioethanol yield of lignocellulosic biomass*.

### **General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal -

### **Take down policy**

If you believe that this document breaches copyright please contact us at [vbn@aub.aau.dk](mailto:vbn@aub.aau.dk) providing details, and we will remove access to the work immediately and investigate your claim.

# OPTIMIZATION OF THE WET EXPLOSION PRETREATMENT FOR INCREASING BIOGAS AND BIOETHANOL YIELD OF LIGNOCELLULOSIC BIOMASS

RAJIB BISWAS



Ph.D. Dissertation  
2012

*A dissertation submitted to the  
Department of Biotechnology, Chemistry and Environmental Engineering  
of Aalborg University in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY.*

— *To my parents, my sisters and my brother  
who did not see me much during the last years...*

*Supervisors:*

HINRICH UELLEND AHL

*Associate Professor*

*Section for Sustainable Biotechnology*

*Aalborg University Copenhagen*

*A C Meyers Vænge 15, 2450 Copenhagen SV*

*Denmark*

&

BIRGITTE K. AHRING

*Director and Battelle Distinguished Professor*

*Center for Bioproducts and Bioenergy*

*Washington State University*

*2710 Crimson Way, Richland, WA 99354*

*USA*



## **Preface**

This dissertation represents a culmination of research work and learning that has taken place over the past few years (2009-2012). The experimental work of this dissertation has been initiated at the Section for Sustainable Biotechnology, Aalborg University Copenhagen, Denmark in September 2009. Since February 2011 until the end of 2012, the rest of the research based on pilot-scale experimental work has been conducted at the Bioproducts, Sciences, and Engineering Laboratory (BSEL) in collaboration with Washington State University (WSU) and the Pacific Northwest National Laboratory (PNNL), USA. The work on this dissertation has been carried out under the supervision of Associate Professor Hinrich Uellendahl and Professor Birgitte K. Ahring.

The dissertation is organized as a short summary (English and Danish) and an introduction in the beginning followed by a collection of journal manuscripts, consisting of a review paper and three research papers, ending with brief concluding remarks. The individual manuscripts are printed in a layout form consistent with the journal for which the individual manuscripts are intended.

### **Paper I**

Wet explosion as a pretreatment method in lignocellulosic biorefineries: status and perspectives

Rajib Biswas, H. Uellendahl and B. K. Ahring

Intended for submission to *Biotechnology Advances*

### **Paper II**

Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure

Rajib Biswas, B. K. Ahring and H. Uellendahl

Published in *Water Science & Technology* (2012), 66 (8), 1751–1758.

### **Paper III**

Wet explosion pretreatment of sugarcane bagasse for enhanced enzymatic hydrolysis

Rajib Biswas, H. Uellendahl and B. K. Ahring

Intended for submission to *Bioresource Technology*

#### **Paper IV**

Effects of inhibitors formed during bagasse pretreatment on ethanol fermentation by  
*Pichia stipitis* CBS6054

Rajib Biswas, H. Uellendahl and B. K. Ahring

Intended for submission to Biomass & Bioenergy

This dissertation has been submitted for assessment in partial fulfillment of the PhD degree. The dissertation is based on the intended for submission or submitted or published scientific papers which are listed above. Parts of the papers are used directly or indirectly in the extended summary of the dissertation. As part of the assessment, co-author statements have been made available to the assessment committee and are also available at the Faculty. The dissertation is not in its present form acceptable for open publication but only in limited and closed circulation as copyright may not be ensured.

Rajib Biswas

r.biswas@tricity.wsu.edu

November 2012, Richland, WA, USA.

## Acknowledgments

The submission of this dissertation brings to an end a wonderful journey filled with many joyous moments, as well as hurdles. I was very fortunate to work with a bunch of extraordinary people at the the Section for Sustainable Biotechnology, Aalborg University Copenhagen, Denmark and at the Bioproducts, Sciences, and Engineering Laboratory (BSEL) in collaboration with Washington State University (WSU) and the Pacific Northwest National Laboratory (PNNL), USA. The dissertation would not have been possible without the help of so many people in so many ways. I sincerely thank all of you who helped directly or indirectly in this study.

I am deeply indebted to my supervisors Hinrich Uellendahl and Birgitte K. Ahring for their guidance, understanding, patience, and most importantly, their friendship during this period. Their mentorship was paramount in providing a well rounded experience consistent my long-term career goals. As you know, my path towards finishing this study was not smooth at all, but you always gave me strength, support and courage to stand up and step forward.

I deeply acknowledge Prof. Bo Svensson and Prof. Jörgen Ejlerthsson at Linköping University, Sweden for introducing me in the field of environmental biotechnology and engineering. Thanks for your assistance and guidance in getting my graduate career started on the right foot and providing me with the foundation for becoming a bioprocess engineer.

A bunch of special thanks goes to my colleagues and friends for assisting me in the lab, at pilot-plant facilities, with proof-reading the manuscripts in spite of their busy schedule, and for the comfort and well-behavior throughout the whole period. I appreciate the way you have supported and shown interest in my work. The discussion with you is always amusing, inspiring and enjoyable.

Finally, my deepest gratitude goes to my family, parents, sisters, brother and friends for their unflagging love, inspiration and strong support throughout my life. You have strengthened me in the darkest times and believed in me even I did not believe in myself. I will always love you all.

*The weak can never forgive.  
Forgiveness is the attribute of the strong...*

[GANDHI, 1869–1948]

## Content

Summary .....	I–III
Summary in Danish .....	IV–VI
 A. Introduction and aim of the PhD study .....	 A.1–A.10
 B. Review paper .....	 B.1–B.41
<i>Wet explosion as a pretreatment method in lignocellulosic biorefineries: status and perspectives</i>	
 C. Research paper I .....	 C.1–C.8
<i>Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure</i>	
 D. Research paper II .....	 D.1–D.19
<i>Wet explosion pretreatment of sugarcane bagasse for enhanced enzymatic hydrolysis</i>	
 E. Research paper III .....	 E.1–E.12
<i>Effects of inhibitors formed during bagasse pretreatment on ethanol fermentation by Pichia stipitis CBS6054</i>	
 Concluding remarks .....	 VII–VIII

## Summary

Production of energy and commodity products such as fuels, high value chemicals and materials from renewable biomass sources has received increased attention in a world of dwindling fossil fuels reserves along with the environmental concerns. While alternative energy sources such as solar, wind, hydropower, and nuclear may play a significant role in powering the globe as a form of electricity to abate greenhouse gas (GHG) emissions, the sole foreseeable sustainable source of carbon based commodity products is biomass. The chemical energy stored in plant materials via photosynthesis can be effectively retrieved exploiting various biotechnological solutions already established. The most widely employed biological processes are fermentation and anaerobic digestion to produce bioethanol and biogas, respectively. Until recently, bioethanol production from sugar- or starch-based feedstocks such as corn, sugarcane, wheat (1<sup>st</sup> generation) has been technologically matured over the last decades. However, due to the controversy over land utilization for production of the 1<sup>st</sup> generation bioethanol, further reconfiguration in the biofuels production is unavoidable. Non-food feedstocks, however, such as lignocellulosic materials including agricultural, municipal and farm wastes, marine biomass and energy crops such as miscanthus and switchgrass (2<sup>nd</sup> generation) are the most scalable alternative sources of biobased products such as biofuels, chemicals and value added commercial products such as xylitol, specialty enzymes, organic acids, single-cell protein in biorefineries. This is because of its accessibility, cheap and environmentally benign production features. Despite the numerous advantages, the microbial conversion of the materials is a major challenge and economical bottleneck to industrial implementation. This is due in large part to the complex structure of the cell wall polymers (that is, cellulose, hemicellulose and lignin) of lignocellulosic biomass, highly recalcitrant to microbial degradation.

In order to improve the rate and extent of hydrolysis of the biomass, disruption of the lignocellulosic matrix to overcome its recalcitrance is crucial. Numerous pretreatment strategies including physical, chemical, thermal, thermochemical, biological have been extensively investigated on lignocellulosic biomass, as one of the necessary steps in lignocellulosic biorefineries. While the pretreatments are effective in improving the hydrolysis, during processing of the biomass various degradation products such as weak acids, 5-hydroxymethylfurfural (HMF), furfural can be formed which are known to inhibit the biological process in higher concentrations. A successful pretreatment should be economically and environmentally sound, highly

productive, feedstock agnostic, and technologically efficient. Besides, it should not only ensure an efficient hydrolysis of the polysaccharides but also limit the formation of the degradation products for ultimate biological conversion processes. Through numerous studies wet explosion (WEx) as a thermochemical pretreatment method has been identified as suitable for a wide variety of lignocellulosic materials. WEx pretreatment uses a hermetically sealed steel reactor to expose biomass to high temperatures, typically 140–210 °C, and pressure (5–35 bar) to solubilize hemicellulose and lignin. Oxygen, air, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), or other oxidizing agents are added to enhance the reaction under high pressure and temperature.

As a part this study, the application of WEx pretreatment has been investigated on residual manure fibers separated after the anaerobic digestion process for enhancing the biogas yield before reintroducing the fiber fraction into the anaerobic digester. This study involves laboratory scale investigation and large scale implementation (FiberMaxBiogas project), various combinations of pretreatment parameters (i.e. 145–180 °C, with/without oxidizing agents) were tested on digested manure fibers. Based on the preliminary results on batch digestion experiments on untreated and wet exploded digested fibers, continuous stirred tank reactors (CSTR) were operated for as long as 214 days. The WEx treatment at 180 °C and a treatment time of 10 min without addition of oxygen was found to be optimal, resulting in 136% increase in methane yield compared with the untreated digested manure fibers in batch experiments. However, the overall methane yield was on average 75% higher than in a control reactor with addition of non-treated digested fibers, when it was tested in continuous mesophilic reactors. Based on the lab-scale results, it was assessed that methane yield per ton of feed could be increased from 23 to 28 m<sup>3</sup> by the recirculation and to 33 m<sup>3</sup> when the biodegradability of the fiber fraction is enhanced from 40 to 75%.

Performances of the WEx pretreatment have been further investigated by conducting pilot-scale experiments on sugarcane bagasse— an ideal substrate for future biorefineries. Sugarcane bagasse is one of the major lignocellulosic materials, rich in glucan and xylan, found in great quantities as a byproduct of sugar and alcohol industries. The temperatures used were 155, 170, 185 and 200 °C with addition of 6 bar oxygen pressure. Similar pretreatments at the target temperatures without oxygen were further performed as a control. Identification and quantification of the degradation products such as acetate, furfural, HMF were carried out along with characterization of the composition of the solid fractions after pretreatment and determination of the concentration of sugars (glucose, xylose, arabinose) released in the liquid phase. Enzymatic

efficiency after pretreatment was significantly improved using 22.0 mg enzyme protein (EP)/g cellulose compared to 12.4 mg EP/g cellulose, tested on washed solid cake. A pretreatment temperature of 185 °C with oxygen yielded the highest sugar concentration in the liquid fraction (34.0 g/L) leaving 59.2% glucan in the solid fraction resulting in a glucose concentration of  $573.9 \pm 4.5$  g/Kg TS and a glucose yield of  $87.4 \pm 0.7\%$  of the theoretical maximum value. The pretreatment carried out at 200 °C with oxygen was not favorable due to lower xylose recovery and formation of the degradation products such as acetate, furfural and HMF of 7.6, 3.3 and 1.0 g/L, respectively, which are known to inhibit microbial growth, although the washed solid samples exhibited enhanced enzymatic efficiency ( $94.8 \pm 0.5\%$ ) under this condition.

Effects of the inhibitors in ethanol fermentation of the WEx exploded bagasse hydrolysates were further investigated using *Pichia stipitis* CBS6054, a native xylose utilizing yeast strain. Sugarcane bagasse used in this study contains glucan, xylan and arabinan of 33.8, 22.0 and 2.4 (% dry weight), respectively. In order to realize industrial cellulosic ethanol production from lignocellulosic biomass, an efficient and suitable pretreatment is the key to facilitate enzymatic hydrolysis and fermentation of the available sugars (both C<sub>6</sub> and C<sub>5</sub>). Initial concentration of the sugars and the degradation products such as acetate, hydroxymethylfurfural (HMF) and furfural were tested with different hydrolysates. The sugar utilization ratio and  $Y_{p/s}$  ranged from 88% to 100% and from 0.33 and  $0.41 \pm 0.02$  g/g, respectively, for all the hydrolysates and controls were tested in this study. The highest cell growth rate for *Pichia stipitis* at 0.09 g/L/h was observed in hydrolysate after wet explosion at 185 °C and 6 bar O<sub>2</sub>, where the hydrolysate was composed of mixed sugars (C<sub>6</sub> and C<sub>5</sub>) and acetate, HMF and furfural concentration of  $3.2 \pm 0.1$ , 0.4 and 0.5 g/L, respectively. *Pichia stipitis* exhibited prolonged fermentation time on bagasse hydrolysate after wet explosion at 200 °C and 6 bar O<sub>2</sub> where the inhibitors concentration was further increased. Nonetheless, ethanol concentration was obtained up to  $18.7 \pm 1.1$  g/L resulting in a yield of  $0.38 \pm 0.02$  g/g after 82 h of fermentation. Almost all of the degradation products (acetate, HMF and furfural) in the hydrolysates was assimilated over time in all samples tested. For the bagasse hydrolysate after wet explosion at 200 °C and 6 bar O<sub>2</sub>, fermentation time is expected to be reduced by increasing the initial cell density and recycling the adapted cells in a continuous process.



## Dansk sammenfatning

I en verden med svindende fossile brændstoffsreserver og øget miljøhensyn har produktion af energi- og råvareprodukter, såsom brændstoffer, højværdikemikalier og materialer fra vedvarende biomasse, fået større og større opmærksomhed. Mens alternative energikilder såsom sol, vind, vandkraft og atomkraft kan spille en væsentlig rolle i at drive kloden i form af elektricitet samt reducere udledningen af drivhusgasser (GHG), er biomasse den eneste bæredygtige kilde til kulstofbaserede råvarer. Den kemiske energi lagret i plantematerialer via fotosyntese kan effektivt udnyttes gennem forskellige bioteknologiske løsninger, der allerede er etableret. De mest anvendte biologiske processer er gæring og anaerob nedbrydning til produktion af hhv. bioethanol og biogas. Produktion af bioethanol fra sukker- eller stivelsesbaserede råmaterialer såsom majs, sukkerrør og hvede (1. generation) er i løbet af de seneste årtier blevet teknologisk modnet. Men, på grund af konflikten om udnyttelse af landarealer til produktion af 1. generations bioethanol er yderligere omkonfiguration i produktion af biobrændstoffer uundgåelig. Ikke-spiselige råmaterialer, såsom lignocellulose materialer fra landbrugs-, kommunalt og gårdaffald, marine biomasse og energiafgrøder, elefantgræs og præriegræs (2. generation), er de mest skalerbare alternativer til produktion af bio-baserede produkter såsom biobrændsel, kemikalier og højværdiprodukter, herunder xylitol, specifikke enzymer, organiske syrer og encellede protein i bioraffinaderier. Dette er på grund af deres tilgængelighed samt billige og miljøvenlige produktionsmetoder. På trods af de mange fordele er den mikrobielle omdannelse af materialerne stadig en stor udfordring samt en økonomisk flaskehals for industriel gennemførelse. Dette skyldes primært den komplekse struktur af cellevægspolymerer (dvs. cellulose, hemicellulose og lignin) i lignocellulose-biomasse, som er meget genstridige over for mikrobiel nedbrydning. For at forbedre hastigheden og graden af hydrolyse af biomasse, er det afgørende at åbne og tilgængeliggøre den lignocelluloseholdige matrix. Talrige forbehandlingsstrategier af lignocellulose-biomasse, herunder fysisk, kemisk, termisk, termokemisk og biologisk, er blevet grundigt undersøgt, som et af de nødvendige skridt i retningen mod lignocellulosebaserede bioraffinaderier. Mens forbehandling er effektiv til at forbedre hydrolyse, kan der under forarbejdningen af biomassepartiklerne dannes forskellige nedbrydningsprodukter, såsom svage syrer, 5-hydroxymethylfurfural (HMF) og furfural som i høje koncentrationer er kendt for at inhibere den efterfølgende biologiske proces. En vellykket forbehandling skal være økonomisk og miljømæssigt rentabel, produktiv, råvare uafhængig og

teknologisk effektiv. Desuden bør den ikke alene sikre en effektiv hydrolyse af polysacchariderne, men også begrænse dannelsen af nedbrydningsprodukter rettet mod den ultimative biologiske omdannelsesproces. Gennem talrige undersøgelser er vådekspllosion (wet explosion, WEx) som en termokemisk forbehandling blevet identificeret som egnet fremgangsmåde til en bred vifte af lignocellulosematerialer. WEx forbehandling benytter en hermetisk lukket stålreaktor til at udsætte biomasse for høje temperaturer, typisk 140 til 210 °C, og tryk (5–35 bar) og derved opløse hemicellulose og lignin. Oxygen, luft, hydrogenperoxid (H<sub>2</sub>O<sub>2</sub>) eller andre oxidationsmidler tilsættes for at forøge reaktionshastigheden under højt tryk og temperatur.

Anvendelse af WEx forbehandling er blevet undersøgt til forøgelse af biogasudbyttet fra gyllefiberrester, hvor fiberrester fra den anaerobe nedbrydningsproces er blevet forbehandlet for derefter at blive genindført i den anaerobe biogasproduktion. Dette studie omfatter laboratorieskala undersøgelser samt storskala implementering (FiberMaxBiogas projektet), hvor forskellige kombinationer af forbehandlingsparametre (145–180 °C, med/uden oxidationsmidler) er blevet testet på fordøjede gyllefibre. Baseret på tidligere resultater fra batch fermenteringsforsøg med ubehandlede og vådeksploderet spaltede fibre, blev kontinuert omrørt reaktorer (continuous stirred tank reactors, CSTR) drevet så længe som 214 dage. WEx forbehandling ved 180 °C og en behandlingstid på 10 min uden tilsætning af oxygen blev fundet til at være optimal, hvilket resulterede i 136% forøgelse af methanudbyttet sammenlignet med de ubehandlede gyllefibre i batch-forsøg. Men, det samlede methan udbytte var i gennemsnit 75% højere end i en kontrol-reaktor med ikke-behandlede fibre, da det blev testet i kontinuert mesofil reaktorer. Baseret på lab-skala resultater blev det vurderet, at methan udbytte per ton foder kan øges fra 23 til 28 m<sup>3</sup> ved recirkulation, og til 33 m<sup>3</sup> når bionedbrydeligheden af fiberfraktionen er øget fra 40 til 75%.

Resultatet af WEx forbehandling, er blevet undersøgt yderligere ved at udføre pilot-skala forsøg på sukkerrørbagasse—et ideelt substrat for fremtidige bioraffinaderier. Sukkerrørbagasse er en af de vigtigste lignocellulosematerialer; det er rigt på glucan og xylan, og det findes i store mængder som et biprodukt fra sukker- og alkoholindustrien. De anvendte temperaturer var 155, 170, 185 og 200 °C med tilsætning af 6 bar oxygentryk. Lignende forbehandlinger ved de specifikke temperaturer, men uden ilt, blev yderligere udført som kontrol. Identifikation og kvantificering af nedbrydningsprodukter, såsom acetat, furfural og HMF blev udført sammen med karakterisering af sammensætningen af de faste fraktioner efter forbehandling og bestemmelse af koncentrationen af sukre (glucose, xylose og arabinose) frigivet i væske-

fasen. Enzymatisk effektivitet efter forbehandling blev signifikant forbedret ved brug af 22,0 mg enzymprotein (EP)/g cellulose sammenlignet med 12,4 mg EP/g cellulose, testet på den vaskede ”faste kage”. En forbehandlingstemperatur på 185 °C med oxygen gav det højeste sukkerindhold i væskefraktionen (34,0 g/L) med 59,2% glukose i den faste fraktion, en glukosekoncentration på  $573,9 \pm 4,5$  g/Kg TS, og et glukoseudbytte af  $87,4 \pm 0,7\%$  af den teoretiske maksimumsværdi. Forbehandling udført ved 200 °C med oxygen var ikke gunstig grundet lavere xylose genvinding samt dannelsen af nedbrydningsprodukter, såsom acetat, furfural og HMF i koncentrationerne hhv 7,6, 3,3 og 1,0 g/L, som er kendt for at inhibere mikrobiel vækst, selv om de vaskede faste prøver viste forøget enzymatisk effektivitet ( $94,8 \pm 0,5\%$ ) under disse omstændigheder.

Effekten af inhibitorer i ethanolfermentering af hydrolysater af WEX eksploderede bagasse blev yderligere undersøgt ved anvendelse af *Pichia stipitis* CBS6054, en gærstamme der naturligt kan fermentere xylose. Den anvendte sukkerrørsbagasse indeholdt glucan, xylan og arabinan på hhv. 33,8, 22,0 og 2,4 (% tørvægt). For at realisere industrielt cellulosebaseret ethanol fra lignocellulose-biomasse er en effektiv og tilpasset forbehandling nøglen til at lette enzymatisk hydrolyse og fermentering af de tilgængelige sukre (både C<sub>6</sub> og C<sub>5</sub>). Oprindelige koncentrationer af sukker- og nedbrydningsprodukter, såsom acetat, hydroxymethylfurfural (HMF) og furfural blev testet for de forskellige hydrolysater. Sukkerudnyttelsesgraden og  $Y_{p/s}$  varierede fra hhv. 88% til 100% og fra 0,33 til  $0,41 \pm 0,02$  g/g for alle hydrolysater og kontroller testet i dette studie. Den højeste cellevæksthastigheden for *Pichia stipitis* blev observeret til at være 0,09 g/L/h i hydrolysateret efter vådekspllosion ved 185 °C og 6 bar O<sub>2</sub>, hvor hydrolysateret er sammensat af blandede sukre (C<sub>6</sub> og C<sub>5</sub>) og acetat, HMF og furfural koncentration på hhv.  $3,2 \pm 0,1$ , 0,4 og 0,5 g/L. *Pichia stipitis* udviste forlænget gæring tid på bagasse hydrolysat efter vådekspllosion ved 200 °C og 6 bar O<sub>2</sub>, hvor inhibitor-koncentrationen var yderligere forøget. Ikke desto mindre blev en ethanol-koncentration på  $18,7 \pm 1,1$  g/L nået, hvilket resulterede i et udbytte på  $0,38 \pm 0,02$  g/g efter 82 timers fermentering. Næsten alle nedbrydningsprodukter (acetat, HMF og furfural) i hydrolysaterne blev forbrugt med tiden i alle testede prøver. For bagasse hydrolysatet efter vådekspllosion ved 200 °C og 6 bar O<sub>2</sub>, forventes fermenteringstiden at blive reduceret ved at forøge den initiale celledensitet og genvinde af de tilpassede celler i en kontinuerlig proces.

## *A. Introduction and aim of the PhD study*

---

In recent years, energy and commodity products such as fuels, high value chemicals and materials from renewable sources has received increased attention in a world of dwindling fossil fuels reserves along with the environmental concerns. The global energy supply is currently dominated by fossil resources (i.e., petroleum, coal, and natural gas), with liquid fuels such as petroleum and petroleum derived-fuels being the longstanding, primary source of energy (Conti and Holtberg, 2011). Exploiting the fossil resources is not only limited to supply transportation fuels but also being utilized as feedstock for variety of other commodity products such as chemicals and materials. However, due to soaring energy prices, volatility of the global oil market, and pressure to abate greenhouse gas (GHG) emissions from fossil fuel consumption, the demand for alternative sources of energy and commodity products had led to invest the attention in its research and development.

While alternative energy sources such as solar, wind, hydropower, and nuclear may play a significant role in powering the globe in the form of electricity, the sole foreseeable sustainable source of carbon based commodity products is biomass (Lynd et al., 2009). Plants capture solar energy through photosynthesis using atmospheric CO<sub>2</sub> and store the energy in the form of chemical energy by building biomass. Often only the chemical energy has been accessed through combustion processes, which usually generate heat and power. The chemical energy stored in plant materials, however, can be retrieved exploiting various biotechnological solutions already established. Besides, biological routes to convert the biomass are preferable over thermochemical platforms such as gasification and pyrolysis, because of their cost effectiveness and potential to achieve nearly theoretical yields (Wyman et al., 2005).

Non-food feedstocks such as lignocellulosic materials including agricultural, municipal and farm wastes, marine biomass and energy crops such as miscanthus and switchgrass hold significant potential (Alvira et al., 2010; Cardona et al., 2010; Lynd et al., 1991, 2009; Rubin, 2008). Utilization of these resources would eliminate the controversy over land utilization for production of the 1<sup>st</sup> generation ethanol from sugar- or starch-based feedstocks such as corn, sugarcane, wheat (Nonhebel, 2005; Tilman et al., 2009; Wheals et al., 1999). Furthermore, production of biofuels and chemicals from the lignocellulosic feedstocks are proven to be the most

scalable alternatives due to their abundance, renewability, technically viable, environmental and socio-economic standpoints (Ahring et al., 1996; Rubin, 2008).

Bioconversion of cellulosic biomass into hydrolyzed sugars is still the major technological and economical bottleneck to industrial implementation (Jørgensen et al., 2007; Yang and Wyman, 2008). More precisely, disruption of the complex cell wall polymers (that is, cellulose, hemicellulose and lignin) is challenging and the structure is highly recalcitrant to microbial degradation. Cellulose is straight chain glucose homopolymer, linked by  $\beta$  - 1,4-glycosidic bonds. It's crystalline structure is strengthened by strong intra- and inter-molecule hydrogen bonds (Figure 1). In contrast, hemicellulose, the second most abundant component of lignocellulose, consists of 5- and 6- carbon ring sugars where xylose is the most common type of sugar. Hemicellulose is highly branched with the presence of acetyl groups, lacking celluloses crystalline structure. Finally, lignin is naturally hydrophobic and amorphous, a complex hydrocarbon polymer composed of three major phenolic components (Rubin, 2008) such as p-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). The existing technology for second generation ethanol, for instance, is not matured enough for commercialization as compared to the first generation industry. This is due in large part to the transformation process that involves biomass collection, size reduction and pretreatment. Each of the steps ultimately facilitates hydrolysis of polysaccharides into sugar monomers that can subsequently be utilized as feedstock for biological conversion. In bioethanol process, usually this hydrolysis is carried out by adding enzyme as a catalyst (enzymatic hydrolysis) to facilitate microbial fermentation of the hydrolyzed sugars. In contrast, in biogas process, hydrolysis is typically mediated by a group of hydrolytic bacteria without any enzyme addition. In order to significantly improve the hydrolysis of lignocellulosic materials for both processes, pretreatment of the biomass is the key bottleneck. This, in addition, will require new technological solutions to improve efficiency by process optimization and to realize industrial implementation (Ahring et al., 1996; Wyman et al., 2005).

For biocoverion processes, pretreatment is considered as the single most expensive process step that hinders the industrial application of lignocellulosic-feedstock-based biorefineries (Alvira et al., 2010; Lynd et al., 1996). In general, the purpose of pretreatment is to disrupt the complex cell wall structure. Besides, pretreatment should help solubilizing the hemicellulose and lignin, and to render the cellulose accessible to facilitate hydrolysis into convertible sugars. Numerous pretreatment methods, including physical (Lin et al., 2010), thermal (Yan et al.,

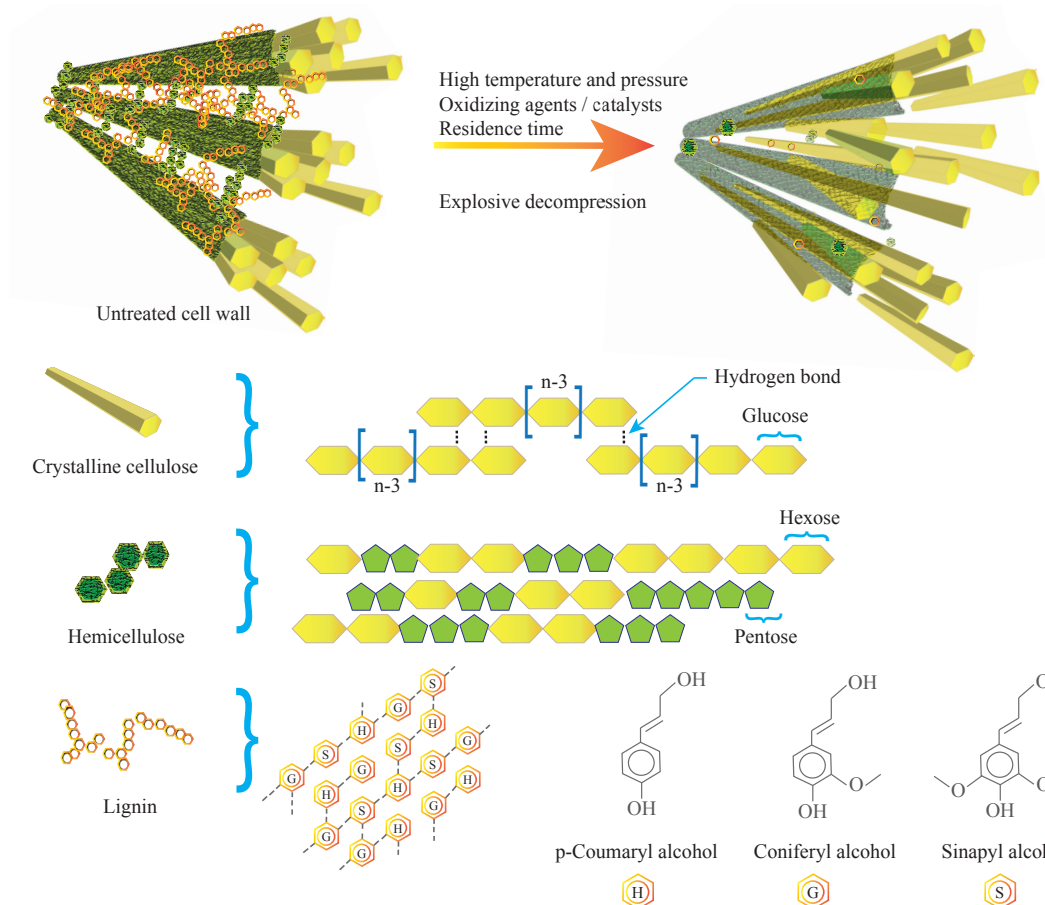


Figure 1: Sketch of wet explosion pretreatment on cell wall structure of lignocellulose as affected by temperature, pressure, oxidizing agents/catalysts, residence time and explosive decompression. Typically the pretreatment solubilizes hemicellulose and a portion of lignin in the aqueous phase while crystalline cellulose remains in the solid fraction. Cellulose, hemicellulose and lignin are the three constituents of lignocellulose and their components are displayed (modified after E. Rubin (Rubin, 2008)).

2009), chemical (Martin et al., 2007), thermochemical (Georgieva et al., 2008), and biological (Singh et al., 2008), have been investigated for last decades. Many of the pretreatment methods have been reviewed and identified as promising for lignocellulosic biorefineries (Alvira et al., 2010; Hendriks and Zeeman, 2009; Mosier et al., 2005; Taherzadeh and Karimi, 2008; Wyman et al., 2005). Of the various pretreatment methods, thermo-chemical pretreatment is considered to be the most suitable for industrial application (Angelidaki et al., 2000; Lissens et al., 2004). This is due to its high conversion rate, no or less chemical use, and thus economically and environmentally sound while applicable to wide range of biomass sources. Of these thermo-chemical conversion methods, wet explosion (WEx), has shown a high potential

for lignocellulosic biomass (Biswas et al., 2012; Georgieva et al., 2008; Rana et al., 2012), and is suitable for a wide variety of biomasses. WEx pretreatment uses a hermetically sealed steel reactor to expose biomass to high temperatures, typically 140-210 °C, and pressure (5-35 bar) to solubilize hemicellulose and lignin (Figure 1). The pretreatment is terminated by explosive decompression to a flash tank (Ahring and Munck, 2006). Oxygen, air, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), or other oxidizing agents (Georgieva et al., 2008) are added to enhance the reaction under high pressure and temperature. In severe pretreatment conditions, the components degradation products such as weak acids, 5-hydroxymethylfurfural, furfural can be formed, that could be inhibitory for subsequent microbial conversion process. Therefore, careful optimization of pretreatment parameters is crucial for every feedstock aiming to maximize the pretreatment effect on hydrolysis and to minimize the formation of the inhibitors.

During my Ph.D. project I studied how abundant lignocellulosic biomass resources can be utilized efficiently as a potential feedstock for the production of biobased products such as biofuels, chemicals and value added commercial products in biorefinery concepts. My focus was on the pretreatment of lignocellulosic biomass for enhancing the hydrolysis of polysaccharides in order to facilitate biological conversion processes in commercial production of biogas and bioethanol.

More concretely, the aim of my PhD study was to optimize wet explosion (WEx) pretreatment parameters for successful hydrolysis of lignocellulosic feedstocks. It was hypothesized that proper adjustment of the wet explosion pretreatment parameters will improve the hydrolysis of lignocellulosics, creating no/tolerable amount of degradation products which are known to inhibit subsequent biological processes, and thus establishing new possibilities towards commercialization of lignocellulosic biorefineries by pilot-scale testing. Since WEx pretreatment has the advantages in applying to a wide variety of biomasses, the technology can be applied to improve hydrolysis of recalcitrant manure fibers and sugarcane bagasse for biogas and bioethanol processes, respectively. The main questions were:

1. What are the promising features that make the wet explosion pretreatment a suitable method for processing lignocellulosic materials compared to other available technologies?
2. What are the most preferable sets of parameters in WEx pretreatment for improving hydrolysis of the manure fibers and bagasse to facilitate subsequent microbial conversion

processes?

3. How should the pretreatment be implemented in relation to different fermentation processes?
4. What are the significant changes in biomass characteristics and components composition before and after performing the pretreatment under different conditions?
5. What will be the impacts on the biological processes in terms of yield and performance while the wet exploded materials are introduced?

My hypothesis for the work has been that the wet explosion pretreatment is suitable as a pretreatment method for production of biogas and bioethanol from lignocellulosics. This dissertation deals with state-of-art and perspective of wet explosion pretreatment for lignocellulosic biorefineries. This study is endeavoring to evaluate various conditions in wet explosion pretreatment of the fibers materials and sugarcane bagasse by characterizing the treated and untreated materials, identifying and quantifying the degradation products and chemicals, and investigating the materials in biological conversion processes to determine the process performance and effects of the degradation products, if any.

Livestock manure, organic waste and plant biomass are the primary feedstocks for production of biogas in anaerobic digestion process. The biogas process has extensively been applied for waste and wastewater treatment for many years, but it has recently gained new attention in terms of reducing greenhouse gas emission, sustainable waste treatment, nutrient recovery as well as renewable energy alternatives to fossil fuels. Especially, biogas production from manure has the highest reduction effect on greenhouse gas emissions compared to other biofuels production processes (Thyø and Wenzel, 2007). Despite the advantages, low methane yield per volume unit of manure is a challenge for the process economy of manure based centralized biogas plant (Gerin et al., 2008). Recent research strives to explain why the yield and productivity can be persistently low. This is due to the recalcitrant fibers fraction in the feedstock that holds more than 50% of the potential which remains unused in traditional anaerobic digestion process (Biswas et al., 2012; Hartmann et al., 2000; Uellendahl et al., 2007). In Denmark, for example, utilization of livestock manure for biogas production has been projected to be increased up to 40% by 2020 from its 5% in 2009, corresponding to an increase in biogas plant capacity by 8-fold (Biswas et al., 2012). In the last years, high biogas yielding industrial wastes (e.g. fish processing and food industries) have been co-digested with manure for an improved process



economy, however, the supply of these wastes is very limited. Thereby, the manure being a sole-substrate, biogas production has to be improved for an economically feasible operation of the current and future biogas plants. Previously, efforts have been made to improve the biodegradability of the crude manure and manure fibers (Uellendahl et al., 2007). One of the major findings in FiberMaxBiogas project was to enhance the biogas production by improving the biodegradability of the fibers fraction in manure. This could be achieved by recirculating the recalcitrant solids fraction (digested fibers), after the solid-liquid separation of the effluent, for further digestion as well as WEx pretreatment of the fibers fraction in manure. Hence, WEx pretreatment of the digested fibers would be more advantageous since the operational costs for treating only the separated digested fibers, which is equivalent to only 10% (w/w) of the total input, are significantly lower than for the pretreatment of the whole reactor feed. However, the WEx pretreatment parameters must be optimized for the specific process in order to avoid any process disturbance/inhibition may cause by the pretreatment. In this study, under the FiberMaxBiogas project that involves laboratory scale investigation and large scale implementation, various combinations of pretreatment parameters have been tested on digested manure fibers. Focus has been given on exploitation of the untapped potential in the fibers fraction by introducing wet explosion pretreatment as an innovative concept to establish a new technological solution that will enable economic operation of biogas process by improving hydrolysis of the fibers fraction resulting higher biogas yield.

In order to study the implication of the wet explosion pretreatment of lignocellulosic biomass, this dissertation further addresses the problem by conducting pilot-scale investigation on sugarcane bagasse— an ideal substrate for future biorefineries. Like most other agricultural residues, sugarcane bagasse consists of approximately 75% polysaccharides which are mainly composed of cellulose and hemicellulose. A low ash content of 1.9% offers numerous advantages compared with others agro-based residues to the bioprocessing industries (Cardona et al., 2010). Sugarcane bagasse is a byproduct generated after the extraction of sucrose from sugarcane (*Saccharum officinarum*) is available on the spot as a potential feedstock for cellulosic biorefineries (Cardona et al., 2010; Pandey et al., 2000), especially in the production of bioethanol and value-added commercial products such as xylitol, specialty enzymes, organic acids, single-cell protein, etc. (Chandel et al., 2012). Despite the huge potential, utilization of bagasse has been limited mostly to heat generation by incineration which accounts for only 50% of the total amount. This is in large part due to the limitation of hydrolysis and requirement for large quan-

ties of cellulase enzymes for saccharification which is disadvantageous for process economy (Pandey et al., 2000). Furthermore, sugarcane bagasse is rich in glucan and xylan (C<sub>6</sub> and C<sub>5</sub> sugars), contains glucan, xylan and arabinan of 33.8, 22.0 and 2.4 (% dry weight), respectively. *Saccharomyces cerevisiae* is the most commonly used yeast for industrial ethanol fermentation, only capable of glucose fermentation. The importance of utilizing all hydrolyzed sugar monomers (that is mostly glucose and xylose in this case) into ethanol for improving process economics is self-evident. Among the xylose fermenting yeasts, *Pichia stipitis* is one of the most promising strains for industrial application due to its high ethanol yield and capable of fermenting most of the sugars glucose, xylose, mannose, galactose and cellobiose (Agbogbo and Coward-Kelly, 2008). Furthermore, it also has the natural ability to metabolize some of the sugar degradation compounds present in the hydrolysate after pretreatment (Almeida et al., 2008; Wan et al., 2012). Efforts have been made in this study to provide an efficient technological solution for improved saccharification of bagasse, investigating enzymatic efficiency by optimizing WEx pretreatment conditions that would further reduce the requirement for large amount of enzymes, could ensure the advanced utilization of the feedstock for biorefineries. The experimental works carried out during this study had been primarily focused on maximizing both C<sub>6</sub> and C<sub>5</sub> sugar yields. This study further investigated the effects of inhibitors on cell growth and ethanol yields of the hydrolysates using *Pichia stipitis*.

This PhD dissertation consists of four papers:

**Paper I:** A review paper that provides brief summary of the basic concepts and parameters involved in WEx and a comparison with other promising pretreatment methods. This also reviews the results in literatures on various thermochemical pretreatment strategies in processing of lignocellulose feedstocks for ethanol and biogas production.

**Paper II:** Original research paper on optimization of wet explosion pretreatment on digested fibers in order to increase methane yields where batch and continuous processes were investigated towards large scale implementation and economic biogas production from manure. The initial approach was to exploit the untapped potential of the digested manure fibers for improving methane yields of manure based biogas plant. The study includes separation of the solid fiber fraction from the effluent of biogas reactor after the primary digestion of manure. The separated solid fraction has then been reintroduced to the reactor with the feed stream after performing the WEx pretreatment. This study involves laboratory scale investigation and large scale implementation, various combinations of pretreatment parameters were tested on digested

manure fibers. Based on the preliminary results from the characterization and batch digestion experiments on determination of methane potential of untreated and wet exploded digested fibers, continuous stirred tank reactors (CSTR) were operated for as long as 214 days. Results obtained from CSTR experiments demonstrate significant improvement in methane yield per volume unit of manure, without any inhibition or process disturbance under the optimized WEx pretreatment of digested fibers.

**Paper III:** Original research paper deals with WEx pretreatment and high solid enzymatic hydrolysis of sugarcane bagasse aiming to optimize the WEx parameters for cost effective hydrolysis to facilitate subsequent fermentation. The experimental works demonstrate the enzymatic efficiency, sugar yields using least amount of enzymes, identification and quantification of the degradation products, effect of pretreatment parameters. This investigation includes pilot scale WEx pretreatment of bagasse under various conditions, solid-liquid separation, characterization of both raw and wet exploded materials by composition analysis.

Finally, **Paper IV:** Original research paper investigates effects of inhibitors on ethanol fermentation of wet exploded bagasse hydrolysate using *Pichia stipitis* CBS6054. Fermentation of wet exploded bagasse hydrolysate containing a mixture of C<sub>5</sub> and C<sub>6</sub> sugars, along with the inhibitors created during the processing of bagasse hydrolysate such as acetate, HMF and furfural were evaluated based on yields, cell growth and productivity.

Although enzyme kinetics and components synergy have been studied further to determine the optimal combinations and ratios of enzymes for the hydrolysis of wet exploded bagasse, the data has not been presented in this dissertation. However, the experiments were designed, for the enzymatic hydrolysis of wet exploded bagasse as presented in this dissertation, based on the preliminary results of the kinetics studies.

## References

- Agbogbo, F., Coward-Kelly, G., 2008. Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. *Biotechnology Letters* 30 (9), 1515–1524.
- Ahring, B., Jensen, K., Nielsen, P., Bjerre, A., Schmidt, A., 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. *Bioresource Technology* 58 (2), 107–113.
- Ahring, B., Munck, J., Mar. 30 2006. Method for treating biomass and organic waste with the purpose of generating desired biologically based products. WO 2006/032282 A1.
- Almeida, J., Modig, T., Röder, A., Lidén, G., Gorwa-Grauslund, M., et al., 2008. *Pichia stipitis* xylose reduc-

- tase helps detoxifying lignocellulosic hydrolysate by reducing 5-hydroxymethyl-furfural (HMF). *Biotechnol Biofuels* 1 (1), 12.
- Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology* 101 (13), 4851–4861.
- Angelidaki, I., Ahring, B., et al., 2000. Methods for increasing the biogas potential from the recalcitrant organic matter contained in manure. *Water Science & Technology* 41 (3), 189–194.
- Biswas, R., Ahring, B., Uellendahl, H., 2012. Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure. *Water Science & Technology* 66 (8), 1751–1758.
- Cardona, C., Quintero, J., Paz, I., 2010. Production of bioethanol from sugarcane bagasse: status and perspectives. *Bioresource Technology* 101 (13), 4754–4766.
- Chandel, A. K., da Silva, S. S., Carvalho, W., Singh, O. V., 2012. Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products. *Journal of Chemical Technology & Biotechnology* 87 (1), 11–20.
- Conti, J., Holtberg, P., 2011. International energy outlook 2011. US Energy Information Administration.
- Georgieva, T., Hou, X., Hilstrøm, T., Ahring, B., 2008. Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading. *Biotechnology for Fuels and Chemicals*, 553–562.
- Gerin, P. A., Vliegen, F., Jossart, J.-M., 2008. Energy and CO<sub>2</sub> balance of maize and grass as energy crops for anaerobic digestion. *Bioresource Technology* 99 (7), 2620 – 2627.
- Hartmann, H., Angelidaki, I., Ahring, B., 2000. Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration. *Water Science & Technology* 41 (3), 145–153.
- Hendriks, A., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology* 100 (1), 10–18.
- Jørgensen, H., Kristensen, J., Felby, C., 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioproducts and Biorefining* 1 (2), 119–134.
- Lin, Z., Huang, H., Zhang, H., Zhang, L., Yan, L., Chen, J., 2010. Ball milling pretreatment of corn stover for enhancing the efficiency of enzymatic hydrolysis. *Applied Biochemistry and Biotechnology* 162 (7), 1872–1880.
- Lissens, G., Klinke, H., Verstraete, W., Ahring, B., Thomsen, A., 2004. Wet oxidation pre-treatment of woody yard waste: parameter optimization and enzymatic digestibility for ethanol production. *Journal of Chemical Technology and Biotechnology* 79 (8), 889–895.
- Lynd, L., Cushman, J., Nichols, R., Wyman, C., 1991. Fuel ethanol from cellulosic biomass. *Science* 251 (4999), 1318–1323.
- Lynd, L., Elamder, R., Wyman, C., 1996. Likely features and costs of mature biomass ethanol technology. *Applied Biochemistry and Biotechnology* 57 (1), 741–761.
- Lynd, L., Larson, E., Greene, N., Laser, M., Sheehan, J., Dale, B., McLaughlin, S., Wang, M., 2009. The role of biomass in america's energy future: framing the analysis. *Biofuels, Bioproducts and Biorefining* 3 (2), 113–123.
- Martin, C., Alriksson, B., Sjöde, A., Nilvebrant, N., Jönsson, L., 2007. Dilute sulfuric acid pretreatment of agricultural and agro-industrial residues for ethanol production. *Applied Biochemistry and Biotechnology* 137 (1),

339–352.

- Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96 (6), 673–686.
- Nonhebel, S., 2005. Renewable energy and food supply: will there be enough land? *Renewable and Sustainable Energy Reviews* 9 (2), 191–201.
- Pandey, A., Soccol, C., Nigam, P., Soccol, V., 2000. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresource Technology* 74 (1), 69–80.
- Rana, D., Rana, V., Ahring, B. K., 2012. Producing high sugar concentrations from loblolly pine using wet explosion pretreatment. *Bioresource Technology* 121 (0), 61–67.
- Rubin, E., 2008. Genomics of cellulosic biofuels. *Nature* 454 (7206), 841–845.
- Singh, P., Suman, A., Tiwari, P., Arya, N., Gaur, A., Shrivastava, A., 2008. Biological pretreatment of sugarcane trash for its conversion to fermentable sugars. *World Journal of Microbiology and Biotechnology* 24 (5), 667–673.
- Taherzadeh, M., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *International Journal of Molecular Sciences* 9 (9), 1621–1651.
- Thyø, K., Wenzel, H., 2007. Life cycle assessment of biogas from maize silage and from manure. Institute for Product Development: Lyngby, Denmark.
- URL [http://www.xergi.com/images/stories/pdf/life\\_cycle\\_assessment\\_report.pdf](http://www.xergi.com/images/stories/pdf/life_cycle_assessment_report.pdf)
- Tilman, D., Socolow, R., Foley, J., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., Somerville, C., et al., 2009. Beneficial biofuels—the food, energy, and environment trilemma. *Science* 325 (5938), 270–271.
- Uellendahl, H., Mladenovska, Z., Ahring, B., 2007. Wet oxidation of crude manure and manure fibers: Substrate characteristics influencing the pretreatment efficiency for increasing the biogas yield of manure. In: *Proceedings 11th World Congress on Anaerobic Digestion*.
- Wan, P., Zhai, D., Wang, Z., Yang, X., Tian, S., 2012. Ethanol Production from Nondetoxified Dilute-Acid Lignocellulosic Hydrolysate by Cocultures of *Saccharomyces cerevisiae* Y5 and *Pichia stipitis* CBS6054. *Biotechnology Research International* 2012.
- Wheals, A., Basso, L., Alves, D., Amorim, H., 1999. Fuel ethanol after 25 years. *Trends in Biotechnology* 17 (12), 482–487.
- Wyman, C., Dale, B., Elander, R., Holtzapple, M., Ladisch, M., Lee, Y., 2005. Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology* 96 (18), 1959–1966.
- Yan, W., Acharjee, T., Coronella, C., Vásquez, V., 2009. Thermal pretreatment of lignocellulosic biomass. *Environmental Progress & Sustainable Energy* 28 (3), 435–440.
- Yang, B., Wyman, C., 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts and Biorefining* 2 (1), 26–40.

***B. Review paper***  
***(Paper I)***

---

*Wet explosion as a pretreatment method in  
lignocellulosic biorefineries: status and perspectives*

RAJIB BISWAS, H. UELLEND AHL AND B. K. AHRING

*(Intended for submission to Biotechnology Advances)*

# Wet explosion as a pretreatment method in lignocellulosic biorefineries: status and perspectives

Rajib Biswas<sup>a,b</sup>, H. Uellendahl<sup>a</sup>, B.K. Ahring<sup>a,b</sup>

<sup>a</sup>*Section for Sustainable Biotechnology, Aalborg University Copenhagen, A. C. Meyers Vænge 15, 2450 Copenhagen SV, Denmark*

<sup>b</sup>*Center for Bioproducts and Bioenergy, Washington State University, 2710 University Drive, Richland, WA 99354-1671, USA (Email: bka@tricity.wsu.edu)*

---

## Abstract

Lignocellulosic biomass is the most abundant resource for suitable production of biobased products such as biofuels and biochemicals for substituting fuels and chemicals based on petroleum. The microbial conversion of this biomass is, however, a major challenge due to the recalcitrant structure of the cell wall polymers of lignocellulosic biomass. In order to make the different fractions bioavailable for the fermentation process, an efficient pretreatment method is the key. Different pretreatment technologies including physical, chemical, thermal, thermochemical, and biological methods have been investigated in order to find an economically and environmentally sound pretreatment, that is highly productive, feedstock agnostic, and technologically efficient. Through numerous studies wet explosion (WEx) as a thermochemical pretreatment method has been identified as suitable for a wide variety of lignocellulosic biomass sources, especially biomass with high lignin content. Presented here is a summary of the basic concepts and parameters involved in WEx. Furthermore, various strategies for implementing thermochemical pretreatment for ethanol and biogas production are reviewed.

**Keywords:** Wet explosion, wet oxidation, thermochemical pretreatment, lignocellulose, hydrolysis, bioethanol and biogas.

---

## Contents

<b>1</b>	<b>Introduction</b>	<b>2</b>
<b>2</b>	<b>Criteria of a suitable pretreatment method</b>	<b>4</b>
<b>3</b>	<b>Structure and composition of lignocellulose</b>	<b>5</b>
<b>4</b>	<b>Wet explosion as a pretreatment method</b>	<b>6</b>
4.1	Process description . . . . .	7
4.2	Effects of wet explosion pretreatment on lignocellulose . . . . .	7
4.2.1	Effects of temperature and pressure . . . . .	7
4.2.2	Autohydrolysis mechanism . . . . .	8
4.2.3	Effects of pH . . . . .	9
4.2.4	Effects of residence time . . . . .	10

November 5, 2012

4.2.5	Effects of oxidizing agent or other catalysts . . . . .	10
<b>5</b>	<b>Other most common pretreatment methods</b>	<b>11</b>
5.1	Steam and liquid hot water pretreatment . . . . .	11
5.2	Acid/alkali pretreatment . . . . .	11
5.3	Ammonia pretreatment . . . . .	12
5.4	Ionic liquid pretreatment . . . . .	13
5.5	Organosolv pretreatment . . . . .	13
5.6	Biological pretreatment . . . . .	13
<b>6</b>	<b>Tailoring the pretreatment to the subsequent conversion process</b>	<b>14</b>
6.1	Pretreatment for enzymatic hydrolysis and ethanol fermentation . . . . .	14
6.2	Pretreatment in combination with anaerobic digestion . . . . .	20
<b>7</b>	<b>Conclusions and perspectives</b>	<b>26</b>

## 1. Introduction

Since the first oil crisis in 1970's, the demand for alternative sources of energy and chemicals have been increased dramatically to ensure the energy security and to abate greenhouse gas (GHG) emissions from fossil fuel consumption. As a consequence, alternative transportation fuels such as bioethanol production from sugar- or starch-based feedstocks such as corn, sugarcane, wheat [1] and biogas production from organic residues and energy crops [2, 3] were focused over the last decades. However, due to the controversy over land utilization for production of the 1<sup>st</sup> generation ethanol, further reconfiguration in the biofuels production is unavoidable [4, 5]. Consequently, lignocellulosic non-food materials including agricultural and forest residues, municipal wastes and also marine biomass have generated tremendous interests as potential feedstock for clean energy and chemicals production [6–12]. Lignocellulosics are considered to be the potential raw materials for biorefineries in production of biofuels, chemicals and value added products (2<sup>nd</sup> generation) because of its accessibility, cheap and environmentally benign production features. In recent years, abundant lignocellulosic biomass resources received tremendous attention for the conversion into biofuels and other bioproducts as a viable option for improving energy security, meeting the environmental demand by reducing greenhouse gas emissions and to enhance the economic development and employment



[13–16]. However, inefficiencies in the conversion processes represent hurdles in large-scale implementation of the biorefining technologies [17–20].

The transformation of the chemical energy stored in biomass into carbon-based materials is the most promising way of achieving alternative, renewable and reliable sources of energy and biochemicals [21, 22]. Biological routes to convert the biomass are preferable over thermochemical platforms such as gasification and pyrolysis, because of their cost effectiveness, environmentally friendly and potential to achieve nearly theoretical yields [23, 24]. Currently, biological processes mostly investigated for biorefineries to convert biomass via fermentation or anaerobic digestion into either ethanol or biogas, respectively [25, 26]. This conversion of lignocellulosic biomass always involves a pretreatment process to disrupt the cell wall polymers (that is, cellulose, hemicellulose and lignin). The pretreatment facilitates hydrolysis of polysaccharides so that the hydrolysate can subsequently be utilized as feedstock for biological conversion. In particular, the initial conversion of biomass into monomeric organic compounds is the key bottleneck in biofuels and biochemical production that will require new technological solutions to improve efficiency [27–29]. The primary purpose of the pretreatment step is to improve the rate and extent of hydrolysis by disrupting the lignocellulosic matrix and overcoming its recalcitrance. Furthermore, the crystalline structure of cellulose should be rendered to make the sugar polymers accessible for enzymatic hydrolysis.

Operational cost is one of the most important factors associated with the pretreatment of lignocellulosic biomass, is often the highest of the whole conversion process. This key step has been described as the least technologically-mature and most expensive step preventing the industrial application of lignocellulosic biorefineries [30–33]. This is due in large part to the nature of the lignocellulosic structure which demands high energy input and lack of optimization of the process on variety of feedstock using current pretreatment methods.

The effects of various physical, biological, chemical and thermal pretreatment methods on the fractionation of lignocellulosic biomass have been evaluated [24, 26, 34–41]. Of the various pretreatment methods, thermochemical pretreatment is considered to be the most suitable for industrial application [42–46]. This is due to its high performance, no or low chemical use, and thus economically and environmentally sound while applicable to wide range of biomass sources [42–45]. The optimization of the pretreatment parameters is crucial for every feedstock source and the subsequent conversion processes. A number of studies [42, 43, 46–63] have been carried out to optimize pretreatment parameters on a wide variety of biomass resources, on both

increasing the sugar release and minimizing the formation of inhibitory compounds [64, 65].

Wet explosion (WEx), a combination of thermal hydrolysis, wet oxidation and steam explosion [48, 50, 66], has shown a high potential as pretreatment for a wide variety of lignocellulosic biomass [44, 48, 50, 60, 61, 67, 68].

This review provides an overview of recent developments in thermochemical pretreatment, focusing on wet explosion of lignocellulosic biomass. Specifically, the adjustment of the wet explosion process for the pretreatment of different biomasses for subsequent biogas and bioethanol production are reviewed. Additionally, wet explosion will be compared to other promising pretreatment methods and the feasibility and limitations of scale-up will be discussed.

## **2. Criteria of a suitable pretreatment method**

Promising pretreatment strategies including using physical [26, 69], chemical [70–72], thermal [26, 73], thermochemical [67, 74–76], and biological [77, 78] are under investigation. Most of the methods described are often a combination of two or more of the above, for example, Yue et al. [79] studied ionic liquid with ball-milling and ultrasound irradiation as a method for pretreatment of holocellulose. Yu et al. [80] studied biological pretreatment of rice hulls in combination with either physical or chemical pretreatment. The primary purpose of a pretreatment is to increase the accessibility of cellulose for subsequent hydrolysis by solubilizing hemicellulose and partially solubilizing lignin. An ideal pretreatment should have high sugar recovery feature, producing no or tolerable amount of inhibitory degradation byproducts for microbial growth. Besides, it should be economically and environmentally sound while maintaining applicability to a wide variety of feedstocks.

Furthermore, the amount of enzymes used in a second generation ethanol plant, for instance, can add up to 50% to the process cost. This expense can be minimized by the use of an efficient pretreatment method [81–83]. Therefore, a pretreatment must be effective enough that a successful saccharification can be carried out using least amount of enzyme. The materials obtained in pretreatment and/or enzymatic hydrolysis undergo biological conversion into products such as ethanol, methane, weak acids and other chemicals. Increases in pretreatment severity may lead to the formation of compounds known to inhibit subsequent enzymatic saccharification and microbial growth. This decreases process yields and productivity [65, 84, 85].

The efficacy of each pretreatment may vary widely depending on the biomass type. The major limitations of a pretreatment method include:

1. High energy and/or chemical input, high costs and inefficient for industrial application.
2. Material losses due to degradation– thus low mass recovery, slow conversion process, limited applicability to a variety of feedstock (softwood, hardwood etc.).
3. Hazardous chemicals use, necessity of chemical recovery, corrosion problems– requiring expensive equipment and maintenance, environmental issues associated with the use of chemicals such as ammonia.
4. Formation of degradation products (inhibitors), detoxification can be necessary for further fermentation.

### **3. Structure and composition of lignocellulose**

Lignocellulose consists of cellulose, hemicellulose and lignin (Figure 1). The composition of lignocellulosic biomass varies depending on the type, origin and variety [86, 87], even within species and particularly between softwood and hardwoods [88]. Cellulose is straight chain glucose homopolymer, linked by  $\beta$  – 1,4–glycosidic bonds ( $C_{6n}H_{10n+2}O_{5n+1}$  (n = degree of polymerization of glucose)). It's crystalline structure is strengthened by strong intra- and inter-molecule hydrogen bonds. In contrast, hemicellulose, the second most abundant component of lignocellulose, consists of 5– and 6– carbon ring sugars where xylose is the most common type of sugar. The other components of hemicellulose are glucose together with arabinose and galactose, and lower levels of rhamnose, mannose, fucose and uronic acids [89]. Hemicellulose is highly branched with the presence of acetyl groups, lacking celluloses crystalline structure. These heteropolymers bind bundles with cellulose fibrils and enhance the stability of the cell wall [90]. Degree of polymerization (DP) of cellulose and hemicellulose ranges from 100-20,000 and 50-300, respectively. The proportion of cellulose and hemicellulose and their characteristics depend upon factors such as biomass type, location, maturity at harvest, climate, growing conditions and storage time after harvest [91–95].

Lignin is a complex hydrocarbon polymer composed of three major phenolic components (phenylpropane units), both aliphatic and aromatic constituents, such as p-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) (Figure 1) [17, 90]. Lignin provides structural strength by cross-linking between the phenylpropane units and cellulose-hemicellulose

fibrils which makes the structure difficult for microbial degradation. Lignin is hydrophobic and amorphous. It is considered to be a thermoplastic polymer exhibiting glass transition and melting temperature of around 90 and 170 °C, respectively [93]. Lignin is the most recalcitrant component of the plant cell wall. Thus removal and/or disruption of its chemical bonds are necessary in order to improve the bioavailability of cellulose and hemicellulose for enzymatic penetration and activity [90]. Table 1 shows the chemical composition of a few potential lignocellulosic feedstocks for biorefineries.

Table 1: Chemical composition of some lignocellulosic materials (dry weight % basis).

Lignocellulosic biomass	Cellulose, %	Hemicellulose, %	Lignin, %	Reference
Corn stover	37-42	20-28	18-22	[96–102]
Sugarcane bagasse	26-50	24-34	10-26	[86, 87, 103–107]
Wheat straw	31-44	22-24	16-24	[22, 96, 108–111]
Hardwood stems	40-45	18-40	18-28	[22, 105, 112]
Softwood stems	34-50	21-35	28-35	[22, 63, 85, 112, 113]
Rice straw	32-41	15-24	10-18	[109, 114–117]
Barley straw	33-40	20-35	8-17	[22, 118–120]
Switchgrass	37-46	29-32	12-19	[112, 121–123]
Energy crops	43-45	24-31	19-12	[122, 123]
Grasses (average) <sup>a</sup>	25-40	25-50	10-30	[105, 123]
Manure solid fibers	8-27	12-22	2-13	[124–127]
Municipal organic waste	21-64	5-22	3-28	[128, 129]

<sup>a</sup>e.g. Reed canarygrass, Smooth brome grass, Tall fescue etc.

#### 4. Wet explosion as a pretreatment method

In general, wet explosion (WEx) includes both physical disruption and a partial chemical degradation of the biomass [48, 67, 130]. The WEx equipment is patented by the Danish company Biogasol ApS [46, 67]. Considerable improvements on the WEx process have been made in the pilot plant facilities at WSU [50, 131], to handle material at up to 35% of dry matter, and results in high sugar yields [50, 132, 133]. Previous studies reveal that the efficacy of the WEx treatment is largely depends upon the lignin content of the lignocellulosic fiber while tested on manure fibers for biogas production [134]. Temperature, residence time and the amount of oxygen added to the process are the most important parameters for the WEx pretreatment method for conversion of biomass into convertible lower molecules [48, 50, 60, 130].

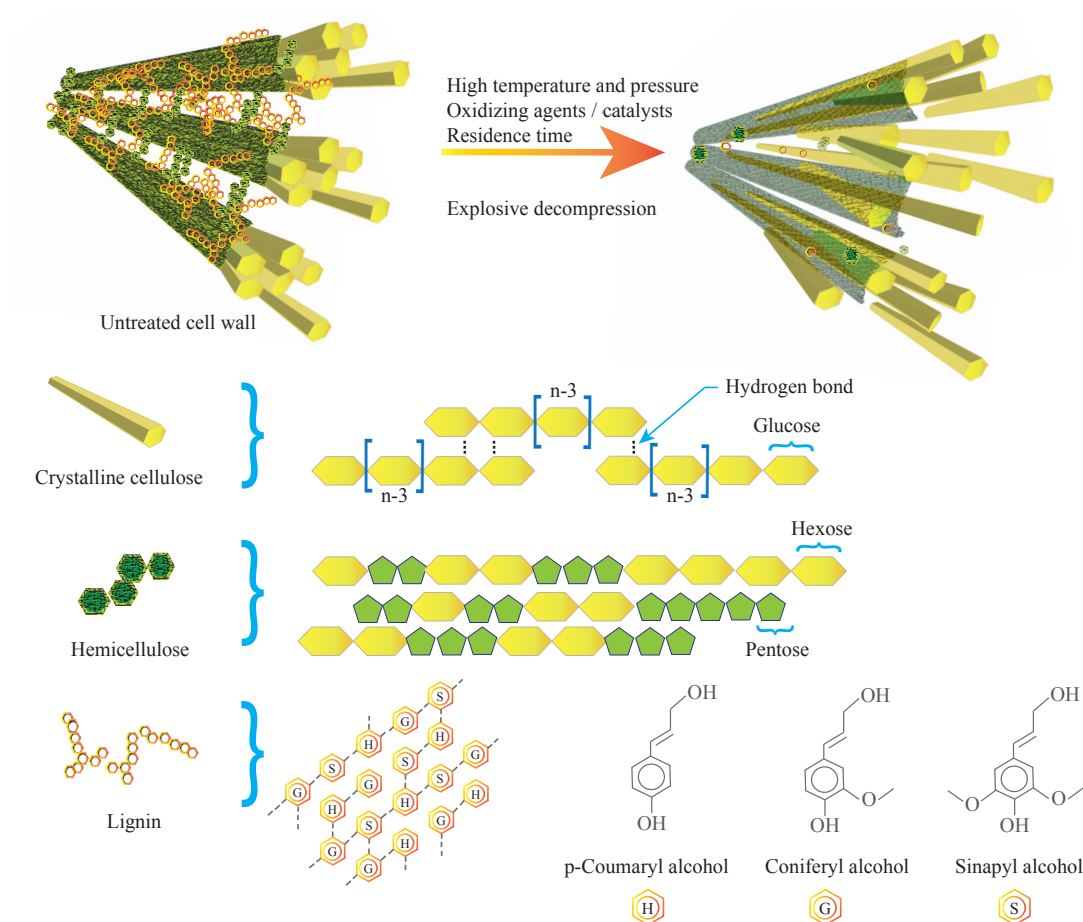


Figure 1: Sketch of wet explosion pretreatment on cell wall structure of lignocellulose as affected by temperature, pressure, oxidizing agents/catalysts, residence time and explosive decompression. Typically the pretreatment solubilizes hemicellulose and a portion of lignin in the aqueous phase while crystalline cellulose remains in the solid fraction. Cellulose, hemicellulose and lignin are the three constituents of lignocellulose and their components are displayed (modified after E. Rubin [17]).

#### 4.1. Process description

WEx pretreatment is performed in a hermetically sealed steel reactor to expose biomass to high temperatures, typically 140-210 °C, and pressure (5-35 bar) to solubilize hemicellulose and lignin, especially when oxygen is added (Figure 2). The reactor is equipped with a stirrer and a flash tank where pretreated biomass slurry is accumulated after the sudden explosive decompression. Wet explosion can be performed with the addition of an oxidizing agent like gaseous oxygen, air, or hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) [46, 48, 130, 135]. To promote hemicellulose and lignin hydrolysis, the treatment is held for a designated period of time and then the materials undergo explosive decompression. The wet exploded material is accumulated in the flash tank and let the flash tank return to atmospheric pressure.

The severity of the pretreatment can be changed by adjustment of the parameters such as temperature pressure, residence time, and the addition of an oxidizing agent. As oxidizing agents, oxygen or air are preferable to other expensive, toxic, corrosive and hazardous chemicals that are under investigation [26, 136]. These oxidants are normally clean for the environment and do not leave residues in the hydrolysate that require additional steps to neutralize or recover the chemical used. Formation of acids is the initial reaction during the oxidation. The solubilization of hemicellulose liberates acids by oxidation of the acidic hemicellulose components and by deesterification of the acetate groups on hemicellulose [128]. This oxidation mechanism further improves the enzymatic hydrolysis with a sugar recovery up to 95% with high enzyme efficiency (Table 2).

#### *4.2. Effects of wet explosion pretreatment on lignocellulose*

##### *4.2.1. Effects of temperature and pressure*

Under WEx conditions, i.e. in an environment that is both oxidizing and acidic, temperature is probably the single most important factor [128] for the disruption of the lignocellulose matrix followed by an oxidizing agent. While the solubilization of hemicellulose starts at 110 °C the cellulosic crystallinity remains unchanged up to 170 °C provided that no oxidative or catalytic agent are added. Typically the effect of temperature is more pronounced while any oxidative agent is added. At temperature above 150 °C autohydrolysis takes place (see section 4.2.2). However, the high temperatures involved in WEx may lead to the formation of degradation products such as weak acids, 5-hydroxymethylfurfural, furfural, which may act as inhibitors in subsequent biological processes [61, 65, 84, 137]. The risk of forming degradation products is increased at higher temperature. This is especially true for nitrogen compounds transformed into oxidative species, while formation of ammonia is observed at temperature below 200 °C [42, 138]. Ammonia can be produced as a stable end-product by oxidation of cyanide and amine-containing compounds [138]. Formation of carboxylic acids (mainly succinic, glycolic, formic and acetic acids) occurs under severe pretreatment conditions as a result of oxidation of sugars, phenols and other compounds [51, 128]. Other sugar degradation products like furfural and hydroxymethyl furfural (HMF) derive from the degradation of pentoses and hexoses, respectively [139].

#### 4.2.2. Autohydrolysis mechanism

During steam explosion pretreatment while applying temperatures typically between 150-230 °C and without acid addition, biomass undergoes autohydrolysis [59, 87, 140, 141]. Autohydrolysis reactions occur at the temperature with or without any oxidizing agents by releasing acetic acid from the esterified form of arabinoxylans, resulting in soluble oligomers due to the depolymerization of hemicellulose polysaccharides [142]. This hydrolysis process is catalyzed by hydronium ions ( $\text{H}_3\text{O}^+$ ) coming from water auto-ionization and also from the acetic acid and uronic acids in latter stage, improving reaction kinetics [143]. During this process, lignin-carbohydrate bonds break down to sugars and phenolic compounds that are soluble in water [110, 144, 145]. Cleavage of acetyl and uronic groups is enhanced with increasing temperature and pressure which liberates acetic and uronic acid, and contributes to a substantial pH drop. Conversely, at mild temperatures ( $>150\text{ }^\circ\text{C}$ ), autohydrolysis does not modify cellulose and lignin substantially, resulting in high-molar mass xyloseoligosaccharides [146]. Thus, the use of proper temperature and pressure for the pretreatment of lognocellulose obviates to some extent the addition of acids. The products of autohydrolysis are a mixture of oligosaccharides, monosaccharides, acetic acids and furan derivatives (furfural and HMF). In addition, furfural and HMF can further undergo decomposition reactions which in turn yield formic and levulinic acid, respectively [143].

#### 4.2.3. Effects of pH

The pH applied in the thermochemical pretreatment influences directly the solubilization of the biomass components [34, 147, 148]. For pretreatments performed under neutral pH conditions, the biomass autohydrolysis tends to lower pretreatment pH (3–4.5) during end of the process, depending on the amount of the acid chains released under the pretreatment conditions (see section 4.2.2). The influence of temperature is more pronounced if the pretreatment is performed under initial acidic condition, improving the solubilization of hemicellulose in particular, leaving the lignin mostly insoluble. On the contrary, lignin solubility increases under alkaline condition due to disruption of lignin structure followed by saponification of intermolecular ester bonds that crosslink hemicelluloses and other components, resulting in increased material porosity [21, 147]. Thermochemical pretreatment of organic waste, for example, showed an increase in COD (chemical oxygen demand) solubilization from 36.9% at pH=8 to 76.1% at pH=13 [148]. Separation of structural linkages between carbohydrate and

lignin results in decreased degree of polymerization and crystallinity. The following equation uses pH to describe the severity factor of a pretreatment [146, 149, 150]:

$$\text{Combined severity (CS)} = \log(R_o) - pH \quad (1)$$

Where,

$$\log(R_o) = \log\{t \times \exp[(T - T_{ref})/14.7]\} \quad (2)$$

Here,  $R_o$  is the severity factor as a function of treatment time ( $t$ , min) and temperature ( $T$ , °C) where  $T_{ref}$  = 100 °C at which no solubilization occurs.

For comparison purposes, pH was proven to be more influential at a temperature of 200 °C and above during hydrothermal liquefaction of biomass [122]. While the formation of degradation products such as 5-hydroxymethyl furfural is favored under acidic conditions, basic conditions result mainly in fragmentation products such as glycolaldehyde and glyceraldehyde. Moreover, the use of an acid catalyst revealed to triple the yield of 5-hydroxymethylfurfural [122].

#### 4.2.4. Effects of residence time

After the pretreatment has reached the desired treatment conditions, i.e. temperature and pressure, the biomass material remains in the reactor for an amount of time which is referred to as the residence time. Residence time is among the key variables affecting the severity of a pretreatment [151, 152].

In general, high temperature activated hydrolysis is fast (<60 minutes) and the residence time largely varies from 2 to 60 minutes depending on the other conditions applied. The pretreatment at higher temperatures requires generally comparatively shorter residence time for achieving a particular effect while lower temperatures need a longer residence time to have the same effect. For example, in steam explosion pretreatment of sweet sorghum bagasse, a 36% higher cellulose conversion was observed at 190 °C when the treatment time was increased from 5 to 10 minutes and 39% improvement was obtained when temperature was switched from 190 to 200 °C with 5 minutes residence time [153]. Arvaniti et al. [45] found 2-3 minutes at 205-210 °C with 12 bar of  $O_2$  gave higher yields and recoveries than that of 15 minutes at



195 °C in wet oxidation of Rape straw. However, the individual effect of residence time is more pronounced under high temperature in terms of hemicellulose-derived sugar recovery in the prehydrolysate [154]. On the other hand, extending the pretreatment time beyond 30 minutes was reported to be disadvantageous due to sugar decomposition reactions as a result of high solid loss [155].

#### *4.2.5. Effects of oxidizing agent or other catalysts*

Delignification of lignin-rich biomass is greatly improved when any oxidizing agent such as compressed atmospheric air or oxygen is added during pretreatment [156]. Cellulose is relatively resistant to oxidizing agents [93], and is only partially solubilized under severe conditions. Nearly complete solubilization of the hemicellulose fraction [157] and partial solubilization of lignin readily occurs when an oxidizing agent is added during pretreatment. The addition of an oxidizing agent offers an advantage compared to acid addition where the lignin is not hydrolyzed [158].

At temperatures above 170 °C, oxygen addition generates an exothermic process resulting in a reduced heating requirement for the pretreatment process [28]. Alternatives to pure oxygen are atmospheric air and H<sub>2</sub>O<sub>2</sub>, although oxygen has proven to be most efficient in lignocellulose convertibility [48]. In addition, O<sub>2</sub> is less costly than H<sub>2</sub>O<sub>2</sub> [48]. Oxidation reactions are two to three times faster in wood biomass solubilization when oxygen was added instead of just air [128], reflecting the influence of higher oxygen concentration. Industrially, oxygen in an aqueous phase has been used in the oxidation of wastes rich in organic matter at high temperature and pressure [159]. However, the presence of oxygen also catalyzes the formation of organic acids and CO<sub>2</sub> from liberated sugars and lignin [42].

### **5. Other most common pretreatment methods**

Although many pretreatment methods are promising for industrial application and have been extensively investigated, generally they offer limited advantages. Some are more suitable for specific biomass type or specific biological process. Besides, formation of inhibitory compounds during pretreatment due to the use of chemicals and the requirement of detoxification prior to the biological process are the key issues. Furthermore, high energy consumption, low mass recovery together with the need for chemical recovery for the process economy are often addressed.

### *5.1. Steam and liquid hot water pretreatment*

Steam pretreatment (also referred to steam explosion) (SP) [160, 161] and liquid hot water (LHW) [162, 163] are two of the most extensively studied pretreatment options. These offer some advantages such as no chemical use, resulting in higher yield of sugars in subsequent enzymatic hydrolysis [38]. Generally the temperature used for LHW is between 150-235 °C, while SP uses 190-270 °C. In steam explosion, explosive decompression using steam is an important feature, as described above for wet explosion (Figure 2). In addition, CO<sub>2</sub> and SO<sub>2</sub> have also been used as catalysts as reported elsewhere [140, 164, 165] and found more cost effective than ammonia [19, 166, 167]. Using LHW method, 2- to 5-fold increase in enzymatic hydrolysis has been reported [64]. LHW is superior in minimizing the formation of degradation products compared to SP [168]. LHW is favored over acid hydrolysis because it is not corrosive while at the same time following similar mechanism to those occurring during acid hydrolysis [64]. However, LHW was also reported to be more energy demanding due to the volume of water required [38, 168] and this method also has less capability in removing lignin [40].

### *5.2. Acid/alkali pretreatment*

Among the acid pretreatment methods available and extensively studied in lignocellulosic biorefineries, dilute acid pretreatment has become probably the most widely used [169–173]. Pretreatments were performed using different kinds of acid such as H<sub>2</sub>SO<sub>4</sub> [169, 174], HCl [165, 175, 176], H<sub>3</sub>PO<sub>4</sub> [177], HNO<sub>3</sub> [178, 179], and usually are carried out at a high temperature (160-230 °C) to enhance effectiveness [155]. However, at ambient temperature (<50 °C) it is more common to use concentrated for hydrolysis [154].

The mechanism of both methods are discussed and reviewed elsewhere [26, 37, 64]. Although concentrated acid pretreatment offers a high rate of cellulose hydrolysis (up to 100%), the necessity for expensive, corrosive resistant equipment; recovery of the hazardous, toxic and corrosive chemical; and the formation of inhibitors, makes the process less attractive [40, 154]. On the other hand, pretreatment with dilute acid requires an enzymatic hydrolysis step to maximize the recovery of fermentable sugars [40] due to the low sugar concentration in the exit stream for the intermediate platform (Figure 2). Hence, dilute acid method is attractive due to its performance in increasing accessible surface area, solubilizing hemicellulose to sugar monomers as well as altering the lignin structure [37]. Nevertheless, risk of degradation product formation, high equipment cost [38] and the use of an alkali to neutralize the hydrolysate

[168] need to be carefully considered when it comes to industrial application [33].

Alkali pretreatments also have a number of challenges. The use of alkali causes swelling of lignocellulose structure which increases the surface area and disrupt the structure of lignin [180]. A number of studies [37, 181, 182] have been carried out to develop and optimize pretreatment using alkali. However, the costs of alkali process increase due to low quality of lignin as a by-product [183]. Furthermore, consumption of alkali by biomass itself [64] necessitate pH adjustment in prior to the pretreated materials undergo any microbial process.

### 5.3. *Ammonia pretreatment*

Ammonia pretreatment such as ammonia fiber explosion (AFEX), ammonia recycled percolation (ARP), and soaking in aqueous ammonia (SAA) have been investigated for lignocelluloses pretreatment [184–187]. In general, this method is a moderate to high temperature and pressure driven physiochemical pretreatment where liquid ammonia is used as catalyst. This process mechanism is similar to wet explosion pretreatment except that liquid ammonia (typically, 1-2 kg of ammonia/kg of DM) is used. However, this pretreatment is reported to be effective only on the lignocellulose with low lignin content, not effective for softwood [22, 40, 154, 165]. The advantages of AFEX are that it does not produce inhibitors for the downstream processes [165], it helps to alter and remove lignin and some hemicellulose while decrystallizing the cellulose. But major drawbacks of ammonia pretreatments include the direct fixed capital cost [38], sophisticated residual ammonia recovery process, limited to biomass-dependent efficiency, high energy consumption, and, most importantly, the environmental issues associated with using ammonia [165].

### 5.4. *Ionic liquid pretreatment*

The use of ionic liquids (ILs) can be very effective in dissolution of cellulose by releasing the hydrogen bonding with no production of inhibitors. Its potential has been tested on varieties of lignocellulosic biomass [188–191]. While promising, high recyclability of the ionic liquid is essential for economic viability [192]. Besides, this method is still in the exploratory stage for industrial scale application. However, for the production of any specific high value chemicals or synthesis of organics such as HMF from glucose using 1-ethyl-3-methylimidazolium chloride, this method is reported to produce yields high enough to compensate for the associate costs [193]. For future lignocellulosic biorefineries, there is a great potential in the development of IL pretreatment [190, 191].

### 5.5. *Organosolv pretreatment*

Organosolv pretreatment uses organic solvent mixtures alone or with an acid or alkaline catalyst to target the internal bonds between lignin and hemicellulose. This method has attracted much attention and demonstrated its potential on a variety of biomasses such as sugarcane bagasse, wheat straw, poplars, beech wood etc. [194–196]. Excellent results have been reported especially for woody biomass [197]. The limitation to the method is that the organic solvent must be recycled in order for the process to be economically acceptable. In addition to that, any leftover organic can be inhibitory for subsequent microbial process [154]. Although this method is costly in comparison to steam explosion, it can produce high-quality reactive lignin and cellulose and an aqueous hemicellulose stream.

### 5.6. *Biological pretreatment*

Biological pretreatment methods primarily apply fungi and/or bacteria capable of producing biomass-degrading enzymes [77, 78]. However, the rate of biological pretreatment is reported to be slow for industrial application and appropriate fungal growth conditions have to be ensured which can be tedious [168]. In addition, the microbes use some of the released sugars for their growth. However, these kinds of pretreatment involve mild conditions, low energy requirements, and as a result low costs, which demonstrates its potential for sustainable application [38].

## **6. Tailoring the pretreatment to the subsequent conversion process**

Adjustment of the pretreatment parameters is very dependent on the subsequent biological process. An efficient hydrolysis of the polysaccharides to sugar monomers (intermediate platform) can be used for a variety of products and chemicals in biorefineries (Figure 2). In ethanol fermentation process, for example, the pretreatment is carried out initially for a successful enzymatic hydrolysis followed by the yeast fermentation. In this case, efficacy of the pretreatment largely depends on mass recovery after pretreatment, enzymatic convertibility while using least amount of enzyme and creating less inhibitors. On the other hand, wet exploded material directly undergoes anaerobic digestion process. Thereby, the performance of a pretreatment is reflected in the biogas yield differences due to the destruction of volatile solids i.e. improved hydrolysis and resulting no process inhibition.

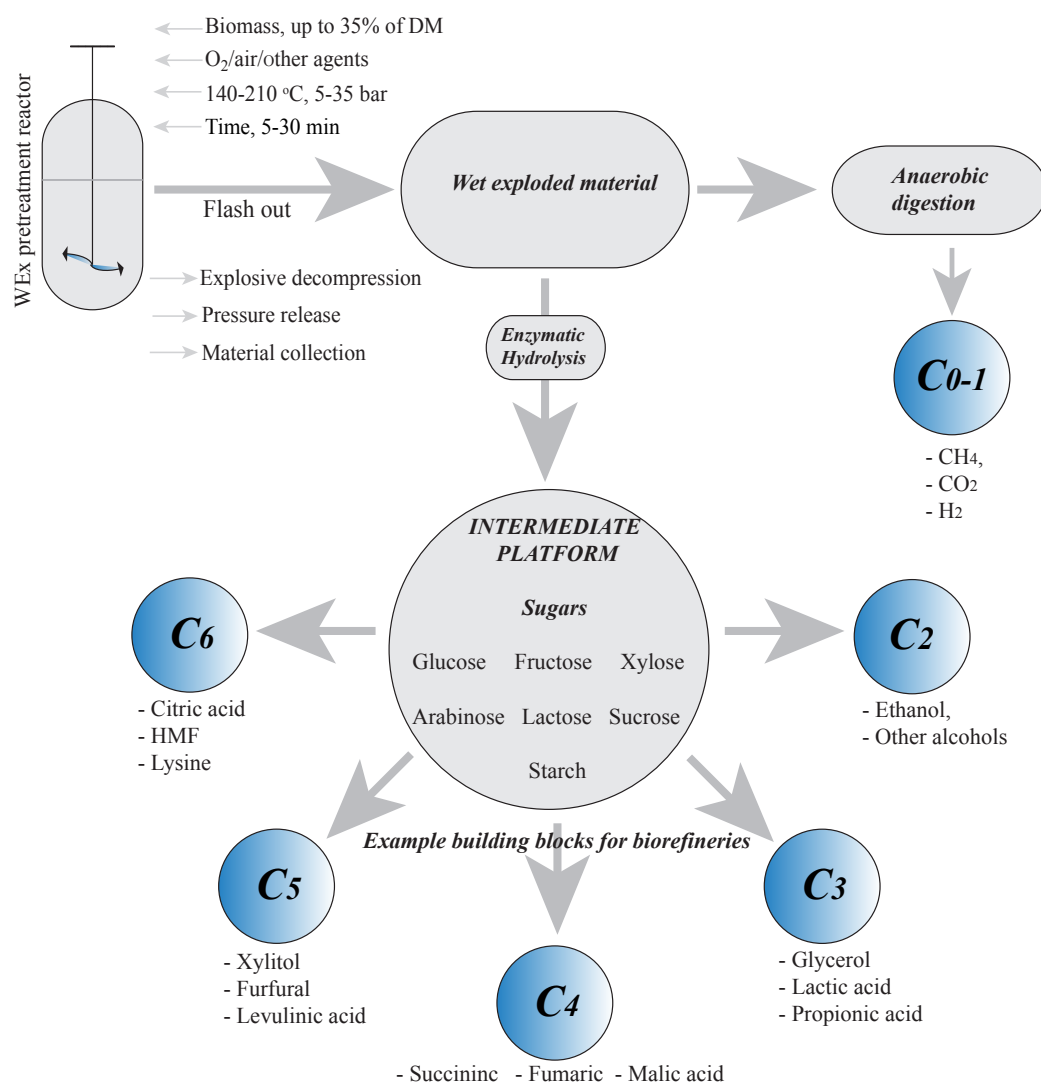


Figure 2: Schematic presentation of wet explosion pretreatment in biorefineries. Pretreatment facilitates enzymatic hydrolysis by liberating sugar monomers for microbial fermentation and also to enhances the anaerobic digestion process. A variety of products can be produced from the sugars using various microbial processes.

### 6.1. Pretreatment for enzymatic hydrolysis and ethanol fermentation

The effects of few promising thermochemical pretreatments that are relevant to be compared to the WEx mechanism, on enzymatic hydrolysis of lignocellulosic feedstocks are compared in Table 2. In particular, the focus was to evaluate pretreatment methods that fully or partially represent the oxidation mechanism with or without an oxidizing agent. Performance of each pretreatment is compared on the basis of the pretreatment conditions applied and the catalyst or agent used to perform hydrolysis. The degree of formation of degradation products in the pre-hydrolysate as well as the material recovery after the pretreatment were considered to be important factors. In order to assess a pretreatment's effect on enzymatic hydrolysis, it is important

to consider factors such as enzyme to dry matter/cellulose ratio as well as enzyme utilization. Moreover, enzymatic convertibility was assessed although number of different equations were used to calculate the enzymatic convertibility. For example, Rana et al. [50] used Eq. 3 to determine the sugar yields of high solids hydrolysis experiments with a correction factor was considered as described by Kristensen et al. [198].

$$Yield (\%) = \frac{[Glu_{EH,L}] + 1.0526 \times [Cel_{EH,L}]}{1.111 \times F_{Cellulose_{RB}} \times [ini.sol]} \times 100\% \times f_{Correction} \quad (3)$$

Where,  $f_{Correction}$  is the yield correction factor at high dry matter,  $[Glu_{EH,L}]$  and  $[Cel_{EH,L}]$  are the glucose and cellobiose concentration (g/L) in enzymatic hydrolysate liquid.  $F_{Cellulose_{RB}}$  is the fraction of cellulose in the raw biomass and  $[ini.sol]$  is the initial solids concentration (g/L) used for enzymatic hydrolysis. To determine the enzymatic efficiency, EE(%) and % Glucose yield, more simplified equations (Eq. 4 & 5) were used by others [70, 131].

$$EE (\%) = \frac{[Glucose], g/L + [Xylose], g/L}{(Solids \times Glucan\% \times 1.11) + (Solids \times Xylan\% \times 1.14), g/L} \times 100\% \quad (4)$$

$$Glucose \text{ yield } (\%) = \frac{[Glucose], g/L}{(Solids \times Glucan\% \times 1.11), g/L} \times 100\% \quad (5)$$

In which [Glucose] and [Xylose] represent the concentrations of the respective sugars after enzymatic hydrolysis; 1.11 and 1.14 are the coefficient of glucose and xylose obtained from glucan and xylan, respectively.

Table 2: Overview of the effects induced by different thermal/thermochemical pretreatments to enhance the enzymatic hydrolysis of various feedstocks for ethanol production as published in the literatures.

Pretreatment type	Biomass	Conditions applied	Catalyst /agent	Inhibitors in the pre-hydrolysate	Recovery after pretreatment	DM, enzyme dosage	Enzymatic convertibility	Reference
Wet explosion	Sugarcane bagasse	185 °C, 10 min, 16% of DM	O <sub>2</sub> , 6 bar	Acetic acid, furfural, HMF at 5.3, 1.7, 0.3 g/L, respectively	Negligible loss	[ <sup>1</sup> ] 8% and 22 mg protein/g cellulose	87.4% cellulose	[131]
Wet explosion	Loblolly pine	170 °C, 22 min, 25% of DM	O <sub>2</sub> , 7.2 bar	Acetic acid, furfural, HMF at 3, 1.5, 0.5 g/L, respectively	Negligible loss	[ <sup>2</sup> ] 25%, 60 mg protein/g cellulose	96% cellulose and nearly 100% hemicellulose	[50]
Wet explosion	Wheat straw	150 °C, 15 min, 12-13% of DM	H <sub>2</sub> SO <sub>4</sub> , 0.3% (w/w)	Carboxylic acids, furfural, HMF at 1.5, 81% 0.3, 0.0 g/100g DM	96% cellulose, 81% hemicellulose	[ <sup>3</sup> ] 5%, 20 mg protein/g VS	80% cellulose	[46]
Wet explosion	Cocksfoot grass	150 °C, 15 min, 12-13% of DM	H <sub>2</sub> SO <sub>4</sub> , 0.3% (w/w)	Carboxylic acids, furfural, HMF at 0.9, 0.1, 0.1 g/100g DM	>95% cellulose, >80% hemicellulose	[ <sup>3</sup> ] 5%, 20 mg protein/g VS	74% cellulose	[46]
Wet explosion	Wheat straw	180-185 °C, 15 min, 14% of DM	O <sub>2</sub> , 12-18 bar	Furfural at 0.2 g/L	93% cellulose, 72% hemicellulose	[ <sup>2</sup> ] 14%, 10 FPU/g cellulose	70% cellulose, 68% hemicellulose	[48]

<sup>1</sup> Enzymatic hydrolysis was carried out on the washed pretreated solid fraction

<sup>2</sup> Enzymatic hydrolysis was carried out on the slurry of the pretreated biomass

<sup>3</sup> Enzymatic hydrolysis was carried out on the separated solid fraction of the pretreated biomass

Table 2: Overview of the effects induced by different thermal/thermochemical pretreatments to enhance the enzymatic hydrolysis of various feedstocks for ethanol production as published in the literatures.

Pretreatment type	Biomass	Conditions applied	Catalyst /agent	Inhibitors in the pre-hydrolysate	Recovery after pretreatment	DM, enzyme dosage	Enzymatic convertibility	Reference
Wet explosion	Wheat straw	180-185 °C, 15 min, 14% of DM	15 H <sub>2</sub> O <sub>2</sub> , 35% (v/v)	Furfural at 0.3 g/L	>90% cellulose, >55% hemicellulose	[ <sup>2</sup> ] 5%, 20 FPU/g cellulose	69% cellulose, 55% hemicellulose	[48]
Wet explosion	Miscanthus <sup>4</sup>	170 °C, 5 min, 15% of DM	Air, <18 bars	No data available	61.3% glucose, 94.9% xylose	[ <sup>2</sup> ] 7%, 45 FPU/g DM & 55 FBG/g DM	56.1% glucose, 31.7% xylose	[130]
Wet explosion	Miscanthus <sup>4</sup>	170 °C, 5 min, 15% of DM	H <sub>2</sub> O <sub>2</sub> , 5.2 g O <sub>2</sub> /g VS	No data available	63.7% glucose, 82.4% xylose	[ <sup>2</sup> ] 7% and 45 FPU/g DM & 55 FBG/g DM	58.5% glucose, 19.2% xylose	[130]
Wet explosion	Winter rye straw	195 °C, 15 min, 6% of DM	O <sub>2</sub> , 12 bar & Na <sub>2</sub> CO <sub>3</sub> , 2g/L	Carboxilic acids, < 5 g/L	87% cellulose, 66% hemicellulose	[ <sup>3</sup> ] 2%, 30 FPU/g DM	49% cellulose, 11% hemicellulose	[199]
Wet explosion	Oilseed rape straw	195 °C, 15 min, 6% of DM	O <sub>2</sub> , 12 bar & Na <sub>2</sub> CO <sub>3</sub> , 2g/L	Carboxilic acids, < 7 g/L	89% cellulose, 60% hemicellulose	[ <sup>3</sup> ] 2%, 30 FPU/g DM	58% cellulose and < 10% hemicellulose	[199]
Wet explosion	Faba bean straw	195 °C, 15 min, 6% of DM	O <sub>2</sub> , 12 bar & Na <sub>2</sub> CO <sub>3</sub> , 2g/L	Carboxilic acids, < 6 g/L	92% cellulose and 69% hemicellulose	[ <sup>3</sup> ] 2%, 30 FPU/g DM	43% cellulose, < 10% hemicellulose	[199]

<sup>4</sup>The biomass was soaked in a 0.75% (v/v) H<sub>2</sub>SO<sub>4</sub> solution (10% DM) at 100 °C for 14 h.



Table 2: Overview of the effects induced by different thermal/thermochemical pretreatments to enhance the enzymatic hydrolysis of various feedstocks for ethanol production as published in the literatures.

Pretreatment type	Biomass	Conditions applied	Catalyst /agent	Inhibitors in the pre-hydrolysate	Recovery after pretreatment	DM, enzyme dosage	Enzymatic convertibility	Reference
Wet oxidation	Sugarcane bagasse	195 °C, 15 min, 5.3% of DM	O <sub>2</sub> , 12 bar & Na <sub>2</sub> CO <sub>3</sub> , 0.2% (w/w)	Formic acid, acetic acid, phenols, furfural, HMF at 2.1, 3.2, 2.7, 0.2, 0 g/L, respectively	100% cellulose, around 90% hemicellulose	[ <sup>1, 2</sup> ] 2%, 25 FPU/g DM	57.4% <sup>1</sup> & 54.3% <sup>2</sup> cellulose, 44.8% <sup>1</sup> & 74.9% <sup>2</sup> xylan	[44]
		195 °C, 15 min, 6% of DM	O <sub>2</sub> , 12 bar	Formic, acetic acid, furfural, phenols at 1.6, 1.9, 0.3, 1.7 g/L, respectively	96% cellulose, 77% hemicellulose, 58% lignin	[ <sup>3</sup> ] 2%, 30 FPU/g DM	45% cellulose	[45]
		205 °C, 3 min, 6% of DM	O <sub>2</sub> , 12 bar	Formic acid, acetic acid, furfural, phenols at 1.0, 1.1, 0.2, 1.5 g/L, respectively	>95% cellulose, 100% hemicellulose, 83% lignin	[ <sup>3</sup> ] 2%, 30 FPU/g DM	60% cellulose	[45]
Wet oxidation	Sugarcane bagasse	195 °C, 15 min, 5.5% of DM	Na <sub>2</sub> CO <sub>3</sub> , 0.2% (w/w)	Carboxylic acids, furfural, HMF, phenols at 6.2, 0.2, 0, 3.9 g/100g bagasse, respectively	>92% cellulose, 48% hemicellulose	[ <sup>1, 2</sup> ] 2%, 25 FPU/g DM	74.9% <sup>1</sup> & 68.9% <sup>2</sup> cellulose, nearly 44% <sup>1</sup> & 75% <sup>2</sup> xylan	[51]

Table 2: Overview of the effects induced by different thermal/thermochemical pretreatments to enhance the enzymatic hydrolysis of various feedstocks for ethanol production as published in the literatures.

Pretreatment type	Biomass	Conditions applied	Catalyst /agent	Inhibitors in the pre-hydrolysate	Recovery after pretreatment	DM, enzyme dosage	Enzymatic convertibility	Reference
Wet oxidation	Clover & ryegrass mixtures	195 °C, 10 min, 6.25% of DM	O <sub>2</sub> , 12 bar	Formic, acetic, glycolic acid, furfural, HMF at 1.4, 1.7, 0.7, 0.3, 0.2 g/L, respectively	>94% cellulose	[ <sup>1, 2</sup> ] 2%, 25 FPU/g DM	93.6% <sup>1</sup> & 88.5% <sup>2</sup> cellulose, 66.1% <sup>1</sup> & 93.3% <sup>2</sup> xylan	[51]
	Wheat straw <sup>5</sup>	190 °C, 250 L/h	H <sub>2</sub> O <sub>2</sub> , 0.5%	No data available	100% cellulose, 80%	[ <sup>3</sup> ] 2%, 30 FPU/g DM	Around 60% cellulose, 40% hemicellulose	[52]
	Wheat straw	185 °C, 15 min, 6% of DM	Na <sub>2</sub> CO <sub>3</sub> , 6.5 g/L	Formic, acetic, glycolic, oxalic, maleic acid at 1.5, 2.1, 1.1, 0.7, 0.2 g/L, respectively	95.8% cellulose, 58.0% hemicellulose, 76.0% overall	[ <sup>3</sup> ] 1%, 1500 NCU/mL & 250 CBU/g	66% cellulose	[59]
Steam explosion	Sugarcane bagasse	205 °C, 10 min, 93.9% of DM	Saturated steam, 40 bar	Formic, acetic acid, phenols, furfural, HMF at 0.4, 1.7, 2.1, 0.6, 0.2 g/L, respectively	87% cellulose, around 80% hemicellulose	[ <sup>1, 2</sup> ] 2%, 25 FPU/g DM	48.9% <sup>1</sup> & 39.0% <sup>2</sup> cellulose, 38.8% <sup>1</sup> & 52.3% <sup>2</sup> xylan	[44]

<sup>5</sup>The cut straw (1–6 cm pieces) was presoaked in water at 80-90 °C for 6 min.

## 6.2. Pretreatment in combination with anaerobic digestion

Substantial research efforts have been made in recent years to improve the anaerobic digestibility of lignocellulosic feedstocks by applying different pretreatment methods [28, 35, 200, 201]. Thermal and thermochemical pretreatments have been widely tested for this purpose. Some results found in literature on testing such thermochemical/thermal pretreatment methods are displayed in Table 3.

Unlike fungus-mediated bioethanol processes, the use of commercial enzymes in an anaerobic digestion process is not necessary due to the presence of hydrolytic microorganism— thereby avoiding enzyme related costs. In anaerobic digestion the hydrolysis of organic polymers is usually carried out by bacterial extracellular enzymes (hydrolases) [202] as well as physico-chemical reactions. Vavilin et al. [203] described a two-phase model for hydrolysis kinetics in such system. In the first phase hydrolytic bacteria cover the surface of solids and enzymes are released to which cleave the polymers into monomers [204]. These hydrolytic, acidogenic and acetogenic bacteria utilize the hydrolysis products for growth and also convert them into intermediate products such as acetate, CO<sub>2</sub> and H<sub>2</sub> that can be further utilized by methanogens to produce CH<sub>4</sub> and CO<sub>2</sub> [205, 206].

The whole degradation process is dependent on the proper performance of each group. The methanogens are often more sensitive to any toxicity (e.g. changes in pH, O<sub>2</sub> contamination, inhibitory compounds such as ammonia) than the other group of microorganisms. Several factors related to the inhibition of microbial consortia are described elsewhere [137, 207, 208]. Formation of several acids in higher concentration due to harsh thermochemical pretreatment methods (Section 4.2) may inhibit microbial processes resulting in a lower yield or even complete cessation of methane production. This can partially be avoided when the pretreatment is carried out on recycled digested solid materials that already been through primary digestion steps [67]. No pretreatment is required for the easily degradable materials present in the substrate. Besides, pretreatment of whole feed material can be very energy intensive.

Accordingly, the performance of thermochemical pretreatment largely depends on the type of biomass used. For example, Lissens et al. found only a 7% increase in methane yields as an effect of wet oxidation pretreatment of raw food waste (Table 3), while a 72% increase was achieved with digested biowaste [209]. On the other hand, Uellendahl et al. observed a 10% decrease in methane yield after wet oxidation of fibers separated from raw pig manure at 170 °C with addition of H<sub>2</sub>O<sub>2</sub> (0.1g/g-DM), while the methane yield increased by 42% when

treating digested manure fibers under the same condition [134]. Biswas et al. [67] achieved a 136% increase in CH<sub>4</sub> yield on digested fibers using wet explosion pretreatment at 180 °C for 10 minutes without addition of any oxidizing agents. A similar investigation on digested solid fractions was conducted by Menardo et al. [210] using autoclavation at 120 °C for 30 minutes and the methane yield was increased by 115%.

Table 3: Overview of the effects of different thermal/thermochemical pretreatments to enhance the anaerobic digestion (AD) of feedstocks for biogas production as published in the literature.

Pretreatment type	Feedstock	Conditions applied	Catalyst /agent	AD conditions	Digester size, feed/inoculum	Yield difference <sup>6</sup>	Comments	Reference
Wet explosion	Digested manure fibers	180 °C, 10 min, 12% of DM	None	Mesophillic, 38 °C	117 mL, 1g VS/25 mL	+136% CH <sub>4</sub>	Only 75% yield increase in CSTR <sup>7</sup>	[67]
Wet explosion	Wheat straw	180 °C, 5 min, 10% of DM	H <sub>2</sub> O <sub>2</sub> , 6g /100g DM	Thermophillic, 55 °C	117 mL, 0.5g DM/20 mL	-11% CH <sub>4</sub>	No yield increase in CSTR	[47]
Wet oxidation	Yard waste	185 °C, 15 min	O <sub>2</sub> , 12 bar and Na <sub>2</sub> CO <sub>3</sub> , 2g/L	Thermophillic, 55 °C, 28 days	100 mL, 0.5g VSS/60 mL	+99% CH <sub>4</sub>	Realized 5 days lag period	[209]
Wet oxidation	Food waste	185 °C, 10 min	O <sub>2</sub> , 12 bar and Na <sub>2</sub> CO <sub>3</sub> , 2g/L	Thermophillic, 55 °C, 28 days	100 mL, 0.5g VSS/60 mL	+7% CH <sub>4</sub>	Realized 5 days lag period	[209]
Wet oxidation	Digested biowaste	185 °C, 15 min	O <sub>2</sub> , 12 bar	Thermophillic, 55 °C, 28 days	100 mL, 0.5g VSS/60 mL	+72% CH <sub>4</sub>	1.5 times higher yields in batch than CSTR	[209]
Wet oxidation	Willow	180 °C, 5 min, 15% of DM	H <sub>2</sub> O <sub>2</sub> , 6%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	+80% CH <sub>4</sub>	No data on CSTR	[211]
Wet oxidation	Miscanthus	180 °C, 5 min, 15% of DM	H <sub>2</sub> O <sub>2</sub> , 6%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	-20% CH <sub>4</sub>	No data on CSTR	[211]

<sup>7</sup>Continuous stirred tank reactors.

<sup>8</sup> No available data.

Table 3: Overview of the effects of different thermal/thermochemical pretreatments to enhance the anaerobic digestion (AD) of feedstocks for biogas production as published in the literature.

Pretreatment type	Feedstock	Conditions applied	Catalyst /agent	AD conditions	Digester size, feed/inoculum	Yield difference <sup>6</sup>	Comments	Reference
Wet oxidation	Miscanthus	180 °C, 8 min, 15% of DM	H <sub>2</sub> O <sub>2</sub> , 6%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	-39% CH <sub>4</sub>	No data on CSTR	[211]
Wet oxidation	Miscanthus	180 °C, 5 min, 20% of DM	H <sub>2</sub> O <sub>2</sub> , 6%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	-29% CH <sub>4</sub>	No data on CSTR	[211]
Wet oxidation	Wheat straw	180 °C, 5 min, 15% of DM	H <sub>2</sub> O <sub>2</sub> , 6%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	-6% CH <sub>4</sub>	No data on CSTR	[211]
Wet oxidation	Wheat straw	180 °C, 5 min, 10% of DM	H <sub>2</sub> O <sub>2</sub> , 6%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	-11% CH <sub>4</sub>	No data on CSTR	[211]
Wet oxidation	Corn stalker	180 °C, 5 min, 15% of DM	H <sub>2</sub> O <sub>2</sub> , 6%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	-36% CH <sub>4</sub>	No data on CSTR	[211]
Wet oxidation	Corn stalker	180 °C, 5 min, 15% of DM	H <sub>2</sub> O <sub>2</sub> , 3%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	-18% CH <sub>4</sub>	No data on CSTR	[211]
Wet oxidation	Corn stalker	180 °C, 5 min, 20% of DM	H <sub>2</sub> O <sub>2</sub> , 3%	Thermophillic, 55 °C, 60 days	NA <sup>8</sup>	-31% CH <sub>4</sub>	No data on CSTR	[211]
Wet Oxidation	Digested fibers	170 °C, NA <sup>8</sup> , 5% of DM	H <sub>2</sub> O <sub>2</sub> , 0.1 g/g-DM	Thermophillic, 55 °C, 16 days	117 mL, 1g VS/20 mL	+42% CH <sub>4</sub>	Assumed 5% VS loss	[134]
Wet Oxidation	Cow manure	170 °C, NA <sup>8</sup> , 8% of DM	H <sub>2</sub> O <sub>2</sub> , 0.1 g/g-DM	Thermophillic, 55 °C, 51 days	117 mL, 1g VS/20 mL	+26% CH <sub>4</sub>	Assumed a 5% VS loss	[134]

Table 3: Overview of the effects of different thermal/thermochemical pretreatments to enhance the anaerobic digestion (AD) of feedstocks for biogas production as published in the literature.

Pretreatment type	Feedstock	Conditions applied	Catalyst /agent	AD conditions	Digester size, feed/inoculum	Yield difference <sup>6</sup>	Comments	Reference
Wet Oxidation	Pig manure	170 °C, NA <sup>8</sup> , 3% of DM	H <sub>2</sub> O <sub>2</sub> , 0.1 g/g-DM	Thermophillic, 55 °C, 51 days	117 mL, 1 g VS/20 mL	-28% CH <sub>4</sub>	Assumed 5% VS loss	[134]
Wet Oxidation	Manure-sludge mixture	170 °C, NA <sup>8</sup> , 5% of DM	H <sub>2</sub> O <sub>2</sub> , 0.1 g/g-DM	Thermophillic, 55 °C, 59 days	117 mL, 1 g VS/20 mL	+14% CH <sub>4</sub>	Assumed 5% VS loss	[134]
Wet Oxidation	Fibers of pig manure	170 °C, NA <sup>8</sup> , 14.5% of DM	H <sub>2</sub> O <sub>2</sub> , 0.1 g/g-DM	Thermophillic, 55 °C, 58 days	117 mL, 1 g VS/20 mL	-10% CH <sub>4</sub>	Assumed 5% VS loss	[134]
Wet oxidation	Chicken feathers	140 °C, 60 min, 10g Carbon/L	air, 20 bar	Mesophillic, 36 °C, 70 days	160 mL, NA <sup>8</sup>	+195% CH <sub>4</sub>	Yield as mL CH <sub>4</sub> /g VSS	[212]
Wet oxidation	Chicken feathers	200 °C, 60 min, 10g Carbon/L	air, 20 bar	Mesophillic, 36 °C, 70 days	160 mL, NA <sup>8</sup>	+50% CH <sub>4</sub>	Yield as mL CH <sub>4</sub> /g VSS	[212]
Wet oxidation	Kraft pulp solids	200 °C, 60 min, 10g Carbon/L	air, 20 bar	Mesophillic, 36 °C, 70 days	160 mL, NA <sup>8</sup>	+100% CH <sub>4</sub>	Yield as mL CH <sub>4</sub> /g VSS	[212]
Wet oxidation	Chicken processing waste	200 °C, 60 min, 10g Carbon/L	air, 20 bar	Mesophillic, 36 °C, 70 days	160 mL, NA <sup>8</sup>	-44% CH <sub>4</sub>	Yield as mL CH <sub>4</sub> /g VSS	[212]
Wet oxidation	Newspaper waste	190 °C, 60 min, 20g/L	1.76g O <sub>2</sub>	Mesophillic, 35 °C, 60 days	2L working volume, 9.96 TCOD (g/L)	88% of cellulose was degraded	59% Conversion efficiency	[62]
Wet oxidation	Winter rye straw	195 °C, 15 min, 6% of DM	O <sub>2</sub> , 12 bar and Na <sub>2</sub> CO <sub>3</sub> , 2g/L	Mesophillic, 42 °C, 67 days	100 mL, 0.3g DM/20 mL	+34% CH <sub>4</sub> , g/100g DM	96% of theoretical yield	[199]

Table 3: Overview of the effects of different thermal/thermochemical pretreatments to enhance the anaerobic digestion (AD) of feedstocks for biogas production as published in the literature.

Pretreatment type	Feedstock	Conditions applied	Catalyst /agent	AD conditions	Digester size, feed/inoculum	Yield difference <sup>6</sup>	Comments	Reference
Thermal (autoclave)	Digested solid fractions	120 °C, 30 min	None	Mesophillic, 40 °C, 56 days	2L, ISR <sup>9</sup> =2 (VS basis)	+115% CH <sub>4</sub>	No data on CSTR	[210]
Thermal (autoclave)	Solid fraction of manures	140 °C, 40 min	None	Thermophillic, 55 °C, 10 days	118 mL, 10g wet solid/26 mL	+57% CH <sub>4</sub>	Only 7% yield increase in CSTR <sup>7</sup>	[57]
Steam explosion	Wheat straw	160 °C, 10 min, 22% of DM	Steam, 20 bar	Mesophillic, 37.5 °C	250 mL, ISR <sup>9</sup> =3 (DM basis)	+14% CH <sub>4</sub>	No data on CSTR	[213]
Steam explosion	Wheat straw	180 °C, 15 min, 22% of DM	Steam, 20 bar	Mesophillic, 37.5 °C	250 mL, ISR <sup>9</sup> =3 (DM basis)	+20% CH <sub>4</sub>	No data on CSTR	[213]

<sup>9</sup>Inoculum to substrate ratio



## 7. Conclusions and perspectives

If lignocellulosic feedstock is to play a dominant role in future biorefineries an efficient pretreatment method will be the key to tapping into the potential of this abundant renewable resource. Using a proven pretreatment like wet explosion, could help achieve this goal. The advantages of using wet explosion pretreatment include: i) higher enzymatic efficiency in hydrolysis of biomass for ethanol process as a part of biorefineries; ii) recovery of oxidizing agent is not necessary, can be effective in reducing enzyme requirement during enzymatic hydrolysis, suitable for the process economy and for the environment; iii) applicable to wide variety of feedstocks and biological processes (i.e. bioethanol and biogas), suitable for industrial application; iv) formation of low/tolerable amount of degradation products for subsequent microbial processes. Optimization of pretreatment parameters, for each unique biomass however, is essential in order to minimize formation of the degradation products and maximize the hydrolysis.

Although there have been many promising achievements in applying other pretreatment methods at laboratory or pilot scale, several challenges remain to be solved. High fixed capital costs, the use of corrosive and hazardous chemicals, and related environmental issues are major obstacles which need to be overcome for successful commercialization. Ionic liquid can be an attractive approach to extract and purify biomass components and produce value-added products while producing no inhibitors, so efforts to reduce the cost of this process are of paramount importance. In the future, we must turn our attention to developing new technological solutions are meant to preserve the environment, while cost efficient to boost the commercialization of 2<sup>nd</sup> generation biofuels and bioproducts.

## References

- [1] A. Wheals, L. Basso, D. Alves, H. Amorim, Fuel ethanol after 25 years, *Trends in Biotechnology* 17 (12) (1999) 482–487.
- [2] P. Börjesson, B. Mattiasson, Biogas as a resource-efficient vehicle fuel, *Trends in Biotechnology* 26 (1) (2008) 7–13.
- [3] T. Sreekrishnan, S. Kohli, V. Rana, et al., Enhancement of biogas production from solid substrates using different techniques—a review, *Bioresource Technology* 95 (1) (2004) 1–10.
- [4] D. Tilman, R. Socolow, J. Foley, J. Hill, E. Larson, L. Lynd, S. Pacala, J. Reilly, T. Searchinger, C. Somerville, et al., Beneficial biofuels—the food, energy, and environment trilemma, *Science* 325 (5938) (2009) 270–271.
- [5] S. Nonhebel, Renewable energy and food supply: will there be enough land?, *Renewable and Sustainable Energy Reviews* 9 (2) (2005) 191–201.
- [6] P. McKendry, Energy production from biomass (part 1): overview of biomass, *Bioresource Technology* 83 (1) (2002) 37–46.
- [7] A. Limayem, S. C. Ricke, Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects, *Progress in Energy and Combustion Science* 38 (4) (2012) 449 – 467.
- [8] Ó. Sánchez, C. Cardona, Trends in biotechnological production of fuel ethanol from different feedstocks, *Bioresource Technology* 99 (13) (2008) 5270–5295.
- [9] L. Lynd, E. Larson, N. Greene, M. Laser, J. Sheehan, B. Dale, S. McLaughlin, M. Wang, The role of biomass in america’s energy future: framing the analysis, *Biofuels, Bioproducts and Biorefining* 3 (2) (2009) 113–123.
- [10] S. Wettstein, D. Alonso, E. Gürbüz, J. Dumesic, A roadmap for conversion of lignocellulosic biomass to chemicals and fuels, *Current Opinion in Chemical Engineering* 1 (3) (2012) 218 – 224.
- [11] A. Margeot, B. Hahn-Hagerdal, M. Edlund, R. Slade, F. Monot, New improvements for lignocellulosic ethanol, *Current opinion in Biotechnology* 20 (3) (2009) 372–380.
- [12] W. Mabey, P. McFarlane, J. Saddler, Biomass availability for lignocellulosic ethanol production, *Biomass and Bioenergy* 35 (11) (2011) 4519–4529.
- [13] L. Olsson, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates for ethanol production, *Enzyme and Microbial Technology* 18 (5) (1996) 312–331.
- [14] X. Fang, Y. Shen, J. Zhao, X. Bao, Y. Qu, Status and prospect of lignocellulosic bioethanol production in china, *Bioresource Technology* 101 (13) (2010) 4814–4819.
- [15] C. Cheng, Y. Lo, K. Lee, D. Lee, C. Lin, J. Chang, Biohydrogen production from lignocellulosic feedstock, *Bioresource Technology* 102 (18) (2011) 8514–8523.
- [16] S. Banerjee, S. Mudliar, R. Sen, B. Giri, D. Satpute, T. Chakrabarti, R. Pandey, Commercializing lignocellulosic bioethanol: technology bottlenecks and possible remedies, *Biofuels, Bioproducts and Biorefining* 4 (1) (2010) 77–93.
- [17] E. Rubin, Genomics of cellulosic biofuels, *Nature* 454 (7206) (2008) 841–845.

- [18] C. Wyman, Ethanol from lignocellulosic biomass: technology, economics, and opportunities, *Bioresource Technology* 50 (1) (1994) 3–15.
- [19] C. Hamelinck, G. Hooijdonk, A. Faaij, Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle-and long-term, *Biomass and Bioenergy* 28 (4) (2005) 384–410.
- [20] M. Galbe, G. Zacchi, Pretreatment of lignocellulosic materials for efficient bioethanol production, *Biofuels* (2007) 41–65.
- [21] D. Chiamonti, M. Prussi, S. Ferrero, L. Oriani, P. Ottonello, P. Torre, F. Cherchi, Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method, *Biomass and Bioenergy* In press (0) (2012) 0–0.
- [22] M. Pedersen, A. Meyer, Lignocellulose pretreatment severity–relating pH to biomatrix opening, *New Biotechnology* 27 (6) (2010) 739–750.
- [23] K. Srirangan, L. Akawi, M. Moo-Young, C. P. Chou, Towards sustainable production of clean energy carriers from biomass resources, *Applied Energy* 100 (0) (2012) 172 – 186, [Clean Energy for Future Generations](#).
- [24] C. Wyman, B. Dale, R. Elander, M. Holtzaple, M. Ladisch, Y. Lee, Coordinated development of leading biomass pretreatment technologies, *Bioresource Technology* 96 (18) (2005) 1959–1966.
- [25] P. McKendry, Energy production from biomass (part 2): conversion technologies, *Bioresource Technology* 83 (1) (2002) 47–54.
- [26] M. Taherzadeh, K. Karimi, Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review, *International Journal of Molecular Sciences* 9 (9) (2008) 1621–1651.
- [27] B. Du, L. Sharma, C. Becker, S. Chen, R. Mowery, G. van Walsum, C. Chambliss, Effect of varying feedstock–pretreatment chemistry combinations on the formation and accumulation of potentially inhibitory degradation products in biomass hydrolysates, *Biotechnology and Bioengineering* 107 (3) (2010) 430–440.
- [28] B. Ahring, K. Jensen, P. Nielsen, A. Bjerre, A. Schmidt, Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria, *Bioresource Technology* 58 (2) (1996) 107–113.
- [29] P. Crutzen, A. Mosier, K. Smith, W. Winiwarter, et al., N<sub>2</sub>O release from agro-biofuel production negates global warming reduction by replacing fossil fuels, *Atmospheric Chemistry and Physics* 8 (2) (2008) 389–395.
- [30] L. Lynd, R. Elander, C. Wyman, Likely features and costs of mature biomass ethanol technology, *Applied Biochemistry and Biotechnology* 57 (1) (1996) 741–761.
- [31] A. Chandel, E. Chan, R. Rudravaram, M. Narasu, L. Rao, P. Ravindra, et al., Economics and environmental impact of bioethanol production technologies: an appraisal, *Biotechnology and Molecular Biology Reviews* 2 (1) (2007) 14–32.
- [32] Y. Zheng, Z. Pan, R. Zhang, Overview of biomass pretreatment for cellulosic ethanol production, *International Journal of Agricultural and Biological Engineering* 2 (3) (2009) 51–68.
- [33] M. Balat, Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review,

Energy Conversion and Management 52 (2) (2011) 858–875.

- [34] M. Galbe, G. Zacchi, Pretreatment: The key to efficient utilization of lignocellulosic materials, *Biomass and Bioenergy* (0) (2012) –.
- [35] H. Carrere, C. Dumas, A. Battimelli, D. Batstone, J. Delgenes, J. Steyer, I. Ferrer, Pretreatment methods to improve sludge anaerobic degradability: A review, *Journal of Hazardous Materials* 183 (1-3) (2010) 1–15.
- [36] K. Stamatelidou, G. Antonopoulou, I. Ntaikou, G. Lyberatos, The effect of physical, chemical, and biological pretreatments of biomass on its anaerobic digestibility and biogas production, *Biogas Production: Pretreatment Methods in Anaerobic Digestion* (2012) 55–90.
- [37] N. Mosier, C. Wyman, B. Dale, R. Elander, Y. Lee, M. Holtzapfel, M. Ladisch, Features of promising technologies for pretreatment of lignocellulosic biomass, *Bioresource Technology* 96 (6) (2005) 673–686.
- [38] G. Brodeur, E. Yau, K. Badal, J. Collier, K. Ramachandran, S. Ramakrishnan, Chemical and physicochemical pretreatment of lignocellulosic biomass: A review, *Enzyme Research* 2011 (2011) 787532.
- [39] L. Björnsson, S. Svensson, S. Monavari, E. Kreuger, G. Zacchi, Pretreatment of non-wood lignocellulosic material, US Patent 20,120,156,727 (Jun. 21 2012).
- [40] P. Alvira, E. Tomás-Pejó, M. Ballesteros, M. Negro, Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review, *Bioresource Technology* 101 (13) (2010) 4851–4861.
- [41] A. A. Modenbach, S. E. Nokes, The use of high-solids loadings in biomass pretreatment: a review, *Biotechnology and Bioengineering* 109 (6) (2012) 1430–1442.
- [42] G. Lissens, H. Klinke, W. Verstraete, B. Ahring, A. Thomsen, Wet oxidation pre-treatment of woody yard waste: parameter optimization and enzymatic digestibility for ethanol production, *Journal of Chemical Technology and Biotechnology* 79 (8) (2004) 889–895.
- [43] I. Angelidaki, B. Ahring, et al., Methods for increasing the biogas potential from the recalcitrant organic matter contained in manure, *Water Science & Technology* 41 (3) (2000) 189–194.
- [44] C. Martín, M. Marcet, A. Thomsen, et al., Comparison between wet oxidation and steam explosion as pretreatment methods for enzymatic hydrolysis of sugarcane bagasse, *BioResources* 3 (3) (2008) 670–683.
- [45] E. Arvaniti, A. B. Bjerre, J. E. Schmidt, Wet oxidation pretreatment of rape straw for ethanol production, *Biomass and Bioenergy* 39 (0) (2012) 94 – 105.
- [46] S. Njoku, B. Ahring, H. Uellendahl, Pretreatment as the crucial step for a cellulosic ethanol biorefinery: Testing the efficiency of wet explosion on different types of biomass, *Bioresource Technology* 124 (0) (2012) 105–110.
- [47] G. Wang, H. Gavala, I. Skiadas, B. Ahring, Wet explosion of wheat straw and codigestion with swine manure: Effect on the methane productivity, *Waste Management* 29 (11) (2009) 2830–2835.
- [48] T. Georgieva, X. Hou, T. Hilstrøm, B. Ahring, Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading, *Biotechnology for Fuels and Chemicals* (2008) 553–562.
- [49] B. Ahring, P. Westermann, Coproduction of bioethanol with other biofuels, *Biofuels* (2007) 289–302.
- [50] D. Rana, V. Rana, B. K. Ahring, Producing high sugar concentrations from loblolly pine using wet explo-

- sion pretreatment, *Bioresource Technology* 121 (0) (2012) 61–67.
- [51] C. Martín, H. Klinke, A. Thomsen, Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse, *Enzyme and Microbial Technology* 40 (3) (2007) 426–432.
  - [52] M. H. Thomsen, A. Thygesen, H. Jørgensen, J. Larsen, B. H. Christensen, A. B. Thomsen, Preliminary results on optimization of pilot scale pretreatment of wheat straw used in coproduction of bioethanol and electricity, in: J. D. McMillan, W. S. Adney, J. R. Mielenz, K. T. Klasson (Eds.), *Twenty-Seventh Symposium on Biotechnology for Fuels and Chemicals*, ABAB Symposium, Humana Press, 2006, pp. 448–460.
  - [53] E. Varga, A. Schmidt, K. Réczey, A. Thomsen, Pretreatment of corn stover using wet oxidation to enhance enzymatic digestibility, *Applied Biochemistry and Biotechnology* 104 (1) (2003) 37–50.
  - [54] P. Westermann, B. Ahring, The biorefinery for production of multiple biofuels, *Biofuels for Fuel Cells: Renewable Energy from Biomass Fermentation* (2005) 194–205.
  - [55] A. Elliott, T. Mahmood, Pretreatment technologies for advancing anaerobic digestion of pulp and paper biotreatment residues, *Water Research* 41 (19) (2007) 4273–4286.
  - [56] H. Hartmann, I. Angelidaki, B. Ahring, Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration, *Water Science & Technology* 41 (3) (2000) 145–153.
  - [57] Z. Mladenovska, H. Hartmann, T. Kvist, M. Sales-Cruz, R. Gani, B. Ahring, et al., Thermal pretreatment of the solid fraction of manure: impact on the biogas reactor performance and microbial community., in: *Anaerobic digestion of solid wastes IV. Selected papers from the IWA 4th International Symposium, Copenhagen, Denmark; 31 August-2 September 2005.*, Vol. 53, IWA Publishing, 2006, pp. 59–67.
  - [58] A. Bjerre, A. Olesen, T. Fernqvist, A. Plöger, A. Schmidt, Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose, *Biotechnology and Bioengineering* 49 (5) (1996) 568–577.
  - [59] A. Schmidt, A.S. & Thomsen, Optimization of wet oxidation pretreatment of wheat straw, *Bioresource Technology* 64 (2) (1998) 139–151.
  - [60] H. Klinke, B. Ahring, A. Schmidt, A. Thomsen, Characterization of degradation products from alkaline wet oxidation of wheat straw, *Bioresource Technology* 82 (1) (2002) 15–26.
  - [61] H. Klinke, L. Olsson, A. Thomsen, B. Ahring, Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: Wet oxidation and fermentation by yeast, *Biotechnology and Bioengineering* 81 (6) (2003) 738–747.
  - [62] T. Fox, M. & Noike, Wet oxidation pretreatment for the increase in anaerobic biodegradability of newspaper waste, *Bioresource Technology* 91 (3) (2004) 273–281.
  - [63] H. Palonen, A. Thomsen, M. Tenkanen, A. Schmidt, L. Viikari, Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood, *Applied Biochemistry and Biotechnology* 117 (1) (2004) 1–17.
  - [64] A. Hendriks, G. Zeeman, Pretreatments to enhance the digestibility of lignocellulosic biomass, *Bioresource Technology* 100 (1) (2009) 10–18.
  - [65] E. Palmqvist, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification, *Bioresource Technology* 74 (1) (2000) 17–24.
  - [66] B. Ahring, J. Munck, Method for treating biomass and organic waste with the purpose of generating desired

- biologically based products, WO 2006/032282 A1 (Mar. 30 2006).
- [67] R. Biswas, B. Ahring, H. Uellendahl, Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure, *Water Science & Technology* 66 (8) (2012) 1751–1758.
  - [68] C. Martín, Y. González, T. Fernández, A. Thomsen, Investigation of cellulose convertibility and ethanolic fermentation of sugarcane bagasse pretreated by wet oxidation and steam explosion, *Journal of Chemical Technology and Biotechnology* 81 (10) (2006) 1669–1677.
  - [69] Z. Lin, H. Huang, H. Zhang, L. Zhang, L. Yan, J. Chen, Ball milling pretreatment of corn stover for enhancing the efficiency of enzymatic hydrolysis, *Applied Biochemistry and Biotechnology* 162 (7) (2010) 1872–1880.
  - [70] Z. Zhu, M. Zhu, Z. Wu, Pretreatment of sugarcane bagasse with  $\text{NH}_4\text{OH-H}_2\text{O}_2$  and ionic liquid for efficient hydrolysis and bioethanol production, *Bioresource Technology* 119 (0) (2012) 199 – 207.
  - [71] C. Martin, B. Alriksson, A. Sjöde, N. Nilvebrant, L. Jönsson, Dilute sulfuric acid pretreatment of agricultural and agro-industrial residues for ethanol production, *Applied Biochemistry and Biotechnology* 137 (1) (2007) 339–352.
  - [72] A. Zhang, C. Liu, R. Sun, Dissolution of cellulose in ionic liquids assisted with ethanol pretreatment, *Advanced Materials Research* 538 (2012) 2429–2433.
  - [73] S. Kumar, U. Kothari, L. Kong, Y. Lee, R. Gupta, Hydrothermal pretreatment of switchgrass and corn stover for production of ethanol and carbon microspheres, *Biomass and Bioenergy* 35 (2) (2011) 956–968.
  - [74] A. Converse, I. Kwarteng, H. Grethlein, H. Ooshima, Kinetics of thermochemical pretreatment of lignocellulosic materials, *Applied Biochemistry and Biotechnology* 20 (1) (1989) 63–78.
  - [75] C. Martín, M. Thomsen, H. Hauggaard-Nielsen, A. BelindaThomsen, Wet oxidation pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation of clover-ryegrass mixtures, *Bioresource Technology* 99 (18) (2008) 8777–8782.
  - [76] C. Martín, A. Thomsen, Wet oxidation pretreatment of lignocellulosic residues of sugarcane, rice, cassava and peanuts for ethanol production, *Journal of Chemical Technology and Biotechnology* 82 (2) (2007) 174–181.
  - [77] P. Singh, A. Suman, P. Tiwari, N. Arya, A. Gaur, A. Shrivastava, Biological pretreatment of sugarcane trash for its conversion to fermentable sugars, *World Journal of Microbiology and Biotechnology* 24 (5) (2008) 667–673.
  - [78] C. Wan, Y. Li, Fungal pretreatment of lignocellulosic biomass, *Biotechnology Advances*.
  - [79] F. Yue, W. Lan, A. Zhang, C. Liu, R. Sun, J. Ye, Dissolution of holocellulose in ionic liquid assisted with ball-milling pretreatment and ultrasound irradiation, *BioResources* 7 (2) (2012) 2199–2208.
  - [80] J. Yu, J. Zhang, J. He, Z. Liu, Z. Yu, Combinations of mild physical or chemical pretreatment with biological pretreatment for enzymatic hydrolysis of rice hull, *Bioresource Technology* 100 (2) (2009) 903–908.
  - [81] M. Galbe, G. Zacchi, A review of the production of ethanol from softwood, *Applied Microbiology and Biotechnology* 59 (2002) 618–628.
  - [82] J. Ruffell, B. Levie, S. Helle, S. Duff, Pretreatment and enzymatic hydrolysis of recovered fibre for ethanol production, *Bioresource Technology* 101 (7) (2010) 2267–2272.

- [83] L. Viikari, J. Vehmaanperä, A. Koivula, Lignocellulosic ethanol: From science to industry, *Biomass and Bioenergy*.
- [84] C. Bellido, S. Bolado, M. Coca, S. Lucas, G. González-Benito, M. T. García-Cubero, Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*, *Bioresource Technology* 102 (23) (2011) 10868–10874.
- [85] S. Larsson, E. Palmqvist, B. Hahn-Hägerdal, C. Tengborg, K. Stenberg, G. Zacchi, N. Nilvebrant, The generation of fermentation inhibitors during dilute acid hydrolysis of softwood, *Enzyme and Microbial Technology* 24 (3-4) (1999) 151–159.
- [86] J. White, W. Catallo, B. Legendre, Biomass pyrolysis kinetics: A comparative critical review with relevant agricultural residue case studies, *Journal of Analytical and Applied Pyrolysis* 91 (1) (2011) 1–33.
- [87] N. Sarkar, S. Ghosh, S. Bannerjee, K. Aikat, Bioethanol production from agricultural wastes: An overview, *Renewable Energy* 37 (1) (2012) 19–27.
- [88] S. Duff, W. Murray, et al., Bioconversion of forest products industry waste cellulose to fuel ethanol: a review., *Bioresource Technology* 55 (1) (1996) 1–33.
- [89] R. Singhal, J. Kennedy, S. Gopalakrishnan, A. Kaczmarek, C. Knill, P. Akmar, Industrial production, processing, and utilization of sago palm-derived products, *Carbohydrate Polymers* 72 (1) (2008) 1–20.
- [90] J. P. van Wyk, Biotechnology and the utilization of biowaste as a resource for bioproduct development, *Trends in Biotechnology* 19 (5) (2001) 172 – 177.
- [91] Y. Percival Zhang, M. Himmel, J. Mielenz, Outlook for cellulase improvement: screening and selection strategies, *Biotechnology Advances* 24 (5) (2006) 452–481.
- [92] H. Abdul Khalil, A. Bhat, A. Ireana Yusra, Green composites from sustainable cellulose nanofibrils: A review, *Carbohydrate Polymers* 87 (2) (2012) 963–979.
- [93] M. John, S. Thomas, Biofibres and biocomposites, *Carbohydrate Polymers* 71 (3) (2008) 343–364.
- [94] A. O’Sullivan, Cellulose: the structure slowly unravels, *Cellulose* 4 (3) (1997) 173–207.
- [95] Y. Zhang, L. Lynd, Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems, *Biotechnology and Bioengineering* 88 (7) (2004) 797–824.
- [96] K. Szulczyk, B. McCarl, G. Cornforth, Market penetration of ethanol, *Renewable and Sustainable Energy Reviews* 14 (1) (2010) 394–403.
- [97] F. Agbogbo, K. Wenger, Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*, *Journal of Industrial Microbiology & Biotechnology* 34 (11) (2007) 723–727.
- [98] T. Kim, J. Kim, C. Sunwoo, Y. Lee, Pretreatment of corn stover by aqueous ammonia, *Bioresource Technology* 90 (1) (2003) 39–47.
- [99] Y. Zhang, S. Ding, J. Mielenz, J. Cui, R. Elander, M. Laser, M. Himmel, J. McMillan, L. Lynd, Fractionating recalcitrant lignocellulose at modest reaction conditions, *Biotechnology and Bioengineering* 97 (2) (2007) 214–223.
- [100] W. Kaar, M. Holtzaple, Using lime pretreatment to facilitate the enzymic hydrolysis of corn stover, *Biomass and Bioenergy* 18 (3) (2000) 189–199.
- [101] S. Chen, Q. Yong, Y. Xu, S. Yu, Dilute sulfuric acid pretreatment and enzymatic hydrolysis of corn stover

- into fermentable sugars, *Advanced Materials Research* 535 (2012) 2462–2468.
- [102] R. Kumar, G. Mago, V. Balan, C. Wyman, Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies, *Bioresource Technology* 100 (17) (2009) 3948–3962.
- [103] S. da Cruz, B. Dien, N. Nichols, B. Saha, M. Cotta, Hydrothermal pretreatment of sugarcane bagasse using response surface methodology improves digestibility and ethanol production by SSF, *Journal of Industrial Microbiology & Biotechnology* (2012) 1–9.
- [104] L. Fuentes, S. Rabelo, R. Filho, A. Costa, Kinetics of lime pretreatment of sugarcane bagasse to enhance enzymatic hydrolysis, *Applied Biochemistry and Biotechnology* 163 (5) (2011) 612–625.
- [105] R. Howard, E. Abotsi, E. Jansen van Rensburg, S. Howard, Lignocellulose biotechnology: issues of bio-conversion and enzyme production, *African Journal of Biotechnology* 2 (12) (2004) 602–619.
- [106] A. Pandey, C. Soccol, P. Nigam, V. Soccol, Biotechnological potential of agro-industrial residues. I: sugarcane bagasse, *Bioresource Technology* 74 (1) (2000) 69–80.
- [107] C. Cardona, J. Quintero, I. Paz, Production of bioethanol from sugarcane bagasse: status and perspectives, *Bioresource Technology* 101 (13) (2010) 4754–4766.
- [108] P. Oleskowicz-Popiel, P. Lisiecki, J. Holm-Nielsen, A. Thomsen, M. Thomsen, Ethanol production from maize silage as lignocellulosic biomass in anaerobically digested and wet-oxidized manure, *Bioresource Technology* 99 (13) (2008) 5327–5334.
- [109] J. V. Dyk, B. Pletschke, A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy, *Biotechnology Advances* (0) (2012) –.
- [110] F. Carneiro, T. Silva-Fernandes, L. Duarte, F. Gírio, Wheat straw autohydrolysis: process optimization and products characterization, *Applied Biochemistry and Biotechnology* 153 (1) (2009) 84–93.
- [111] M. Petersen, J. Larsen, M. Thomsen, Optimization of hydrothermal pretreatment of wheat straw for production of bioethanol at low water consumption without addition of chemicals, *Biomass and Bioenergy* 33 (5) (2009) 834–840.
- [112] M. Robbins, G. Evans, J. Valentine, I. Donnison, G. Allison, New opportunities for the exploitation of energy crops by thermochemical conversion in Northern Europe and the UK, *Progress in Energy and Combustion Science* 38 (2) (2012) 138 – 155.
- [113] C. Olsen, V. Arantes, J. Saddler, The use of predictive models to optimize sugar recovery obtained after the steam pre-treatment of softwoods, *Biofuels, Bioproducts and Biorefining* 6 (5) (2012) 534–548.
- [114] K. Chang, J. Thitikorn-amorn, J. Hsieh, B. Ou, S. Chen, K. Ratanakhanokchai, P. Huang, S. Chen, Enhanced enzymatic conversion with freeze pretreatment of rice straw, *Biomass and Bioenergy* 35 (1) (2011) 90–95.
- [115] W. Chen, B. Pen, C. Yu, W. Hwang, Pretreatment efficiency and structural characterization of rice straw by an integrated process of dilute-acid and steam explosion for bioethanol production, *Bioresource Technology* 102 (3) (2011) 2916–2924.
- [116] T. Nguyen, K. Kim, S. Han, H. Cho, J. Kim, S. Park, J. Park, S. Sim, Pretreatment of rice straw with



- ammonia and ionic liquid for lignocellulose conversion to fermentable sugars, *Bioresource Technology* 101 (19) (2010) 7432–7438.
- [117] A. Singh, S. Tuteja, N. Singh, N. Bishnoi, Enhanced saccharification of rice straw and hull by microwave-alkali pretreatment and lignocellulolytic enzyme production, *Bioresource Technology* 102 (2) (2011) 1773–1782.
- [118] B. Saha, M. Cotta, Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol, *New Biotechnology* 27 (1) (2010) 10–16.
- [119] P. Adapa, L. Tabil, G. Schoenau, Compaction characteristics of barley, canola, oat and wheat straw, *Biosystems Engineering* 104 (3) (2009) 335–344.
- [120] A. Jin, J. Ren, F. Peng, F. Xu, G. Zhou, R. Sun, J. Kennedy, Comparative characterization of degraded and non-degradative hemicelluloses from barley straw and maize stems: Composition, structure, and thermal properties, *Carbohydrate Polymers* 78 (3) (2009) 609–619.
- [121] C. Sánchez, Lignocellulosic residues: biodegradation and bioconversion by fungi, *Biotechnology Advances* 27 (2) (2009) 185–194.
- [122] S. S. Toor, L. Rosendahl, A. Rudolf, Hydrothermal liquefaction of biomass: A review of subcritical water technologies, *Energy* 36 (5) (2011) 2328 – 2342.
- [123] D. O. Otieno, B. K. Ahring, The potential for oligosaccharide production from the hemicellulose fraction of biomasses through pretreatment processes: xylooligosaccharides (XOS), arabinooligosaccharides (AOS), and mannoooligosaccharides (MOS), *Carbohydrate Research* 360 (0) (2012) 84 – 92.
- [124] S. Chen, W. Liao, C. Liu, D. Elliott, M. Brown, A. Solana, P. N. N. L. (US), W. S. University, U. S. D. of Energy, Value-added chemicals from animal manure, Pacific Northwest National Laboratory, 2003.
- [125] P. Champagne, Feasibility of producing bio-ethanol from waste residues: a canadian perspective: feasibility of producing bio-ethanol from waste residues in canada, *Resources, Conservation and Recycling* 50 (3) (2007) 211–230.
- [126] S. Chen, Z. Wen, W. Liao, C. Liu, R. Kincaid, J. Harrison, D. Elliott, M. Brown, D. Stevens, Studies into using manure in a biorefinery concept, *Applied Biochemistry and Biotechnology* 124 (1) (2005) 999–1015.
- [127] Z. Wen, W. Liao, S. Chen, Hydrolysis of animal manure lignocellulosics for reducing sugar production, *Bioresource Technology* 91 (1) (2004) 31–39.
- [128] G. McGinnis, W. Wilson, C. Mullen, Biomass pretreatment with water and high-pressure oxygen. The wet-oxidation process, *Industrial & Engineering Chemistry Product Research and Development* 22 (2) (1983) 352–357.
- [129] J. Shi, M. Ebrik, B. Yang, C. Wyman, The potential of cellulosic ethanol production from municipal solid waste: A technical and economic evaluation, *University of California Energy Institute* 0 (2009) 0.
- [130] A. Sørensen, P. Teller, T. Hilstrøm, B. Ahring, Hydrolysis of miscanthus for bioethanol production using dilute acid presoaking combined with wet explosion pre-treatment and enzymatic treatment, *Bioresource Technology* 99 (14) (2008) 6602–6607.
- [131] R. Biswas, H. Uellendahl, B. Ahring, Wet explosion pretreatment of sugarcane bagasse for enhanced enzymatic hydrolysis, In preparation.

- [132] B. Ahring, N. Langvad, et al., Sustainable low cost production of lignocellulosic bioethanol-” the carbon slaughterhouse”. a process concept developed by biogasol., *International Sugar Journal* 110 (1311) (2008) 184–190.
- [133] B. Ahring, J. Munck, Method for treating biomass and organic waste with the purpose of generating desired biologically based products, EP Patent 1,797,203 (Jun. 20 2007).
- [134] H. Uellendahl, Z. Mladenovska, B. Ahring, Wet oxidation of crude manure and manure fibers: Substrate characteristics influencing the pretreatment efficiency for increasing the biogas yield of manure, in: *Proceedings 11th World Congress on Anaerobic Digestion*, 2007.
- [135] T. Georgieva, M. Mikkelsen, B. Ahring, Ethanol production from wet-exploded wheat straw hydrolysate by thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor, *Biotechnology for Fuels and Chemicals* (2008) 99–110.
- [136] E. Sendich, M. Laser, S. Kim, H. Alizadeh, L. Laureano-Perez, B. Dale, L. Lynd, Recent process improvements for the ammonia fiber expansion (AFEX) process and resulting reductions in minimum ethanol selling price, *Bioresource Technology* 99 (17) (2008) 8429–8435.
- [137] Y. Chen, J. Cheng, K. Creamer, Inhibition of anaerobic digestion process: A review, *Bioresource Technology* 99 (10) (2008) 4044–4064.
- [138] S. Kolaczowski, P. Plucinski, F. Beltran, F. Rivas, D. McLurgh, Wet air oxidation: a review of process technologies and aspects in reactor design, *Chemical Engineering Journal* 73 (2) (1999) 143–160.
- [139] H. Klinke, A. Thomsen, B. Ahring, Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass, *Applied Microbiology and Biotechnology* 66 (1) (2004) 10–26.
- [140] M. Balat, H. Balat, C. Öz, Progress in bioethanol processing, *Progress in Energy and Combustion Science* 34 (5) (2008) 551–573.
- [141] D. Chiaramonti, A. Rizzo, M. Prussi, S. Tedeschi, F. Zimbardi, G. Braccio, E. Viola, P. Pardelli, 2nd generation lignocellulosic bioethanol: is torrefaction a possible approach to biomass pretreatment?, *Biomass Conversion and Biorefinery* 1 (1) (2011) 9–15.
- [142] A. J. Esfahlan, R. Jamei, R. J. Esfahlan, The importance of almond (*Prunus amygdalus* L.) and its by-products, *Food Chemistry* 120 (2) (2010) 349 – 360.
- [143] F. Carvalheiro, L. Duarte, F. Gírio, Hemicellulose biorefineries: a review on biomass pretreatments, *Journal of Scientific & Industrial Research* 67 (2008) 849–864.
- [144] G. Rodríguez, A. Lama, R. Rodríguez, A. Jiménez, R. Guillén, J. Fernández-Bolaños, Olive stone an attractive source of bioactive and valuable compounds, *Bioresource Technology* 99 (13) (2008) 5261–5269.
- [145] J. Xu, Z. Wang, J. Cheng, et al., Bermuda grass as feedstock for biofuel production: a review., *Bioresource Technology* 102 (17) (2011) 7613–7620.
- [146] D. Montane, X. Farriol, J. Salvadó, P. Jollez, E. Chornet, Fractionation of wheat straw by steam-explosion pretreatment and alkali delignification. cellulose pulp and byproducts from hemicellulose and lignin, *Journal of Wood Chemistry and Technology* 18 (2) (1998) 171–191.
- [147] M. Pedersen, K. Johansen, A. Meyer, Low temperature lignocellulose pretreatment: effects and interac-

- tions of pretreatment pH are critical for maximizing enzymatic monosaccharide yields from wheat straw, *Biotechnology for Biofuels* 4 (1) (2011) 1–10.
- [148] J. Delgens, V. Penaud, M. Torrijos, R. Moletta, Investigations on the changes in anaerobic biodegradability and biotoxicity of an industrial microbial biomass induced by a thermochemical pretreatment, *Water Science and Technology* 41 (3) (2000) 137–144.
- [149] H. Chum, D. Johnson, S. Black, R. Overend, Pretreatment-catalyst effects and the combined severity parameter, *Applied Biochemistry and Biotechnology* 24 (1) (1990) 1–14.
- [150] N. Brosse, P. Sannigrahi, A. Ragauskas, Pretreatment of *Miscanthus x giganteus* using the ethanol organosolv process for ethanol production, *Industrial & Engineering Chemistry Research* 48 (18) (2009) 8328–8334.
- [151] I. Dogaris, S. Karapati, D. Mamma, E. Kalogeris, D. Kekos, Hydrothermal processing and enzymatic hydrolysis of sorghum bagasse for fermentable carbohydrates production, *Bioresource Technology* 100 (24) (2009) 6543 – 6549.
- [152] W. Kaar, C. Gutierrez, C. Kinoshita, Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol, *Biomass and Bioenergy* 14 (3) (1998) 277 – 287.
- [153] B. Sipos, J. Rcezy, Z. Somorai, Z. Kdr, D. Dienes, K. Rcezy, Sweet sorghum as feedstock for ethanol production: Enzymatic hydrolysis of steam-pretreated bagasse, *Applied Biochemistry and Biotechnology* 153 (2009) 151–162.
- [154] P. Kumar, D. Barrett, M. Delwiche, P. Stroeve, Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production, *Industrial & Engineering Chemistry Research* 48 (8) (2009) 3713–3729.
- [155] A. Redding, Z. Wang, D. Keshwani, J. Cheng, High temperature dilute acid pretreatment of coastal bermuda grass for enzymatic hydrolysis, *Bioresource Technology* 102 (2) (2011) 1415–1424.
- [156] V. Chang, M. Holtzapple, Fundamental factors affecting biomass enzymatic reactivity, *Applied Biochemistry and Biotechnology* 84 (1) (2000) 5–37.
- [157] C. Martín, H. Klinke, M. Marcet, L. García, E. Hernandez, A. Thomsen, Study of the phenolic compounds formed during pretreatment of sugarcane bagasse by wet oxidation and steam explosion, *Holzforschung* 61 (5) (2007) 483–487.
- [158] A. Mohanty, M. Misra, G. Hinrichsen, Biofibres, biodegradable polymers and biocomposites: An overview, *Macromolecular Materials and Engineering* 276 (1) (2000) 1–24.
- [159] H. Jørgensen, J. Kristensen, C. Felby, Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities, *Biofuels, Bioproducts and Biorefining* 1 (2) (2007) 119–134.
- [160] P. Manzanares, I. Ballesteros, M. Negro, J. Oliva, A. Gonzalez, M. Ballesteros, Biological conversion of forage sorghum biomass to ethanol by steam explosion pretreatment and simultaneous hydrolysis and fermentation at high solid content, *Biomass Conversion and Biorefinery* (2012) 1–10.
- [161] G. Rocha, C. Martín, V. da Silva, E. Gómez, A. Gonçalves, Mass balance of pilot-scale pretreatment of sugarcane bagasse by steam explosion followed by alkaline delignification, *Bioresource Technology* 111 (0) (2012) 447 – 452.

- [162] C. S. Goh, H. T. Tan, K. T. Lee, Pretreatment of oil palm frond using hot compressed water: An evaluation of compositional changes and pulp digestibility using severity factors, *Bioresource Technology* 110 (0) (2012) 662 – 669.
- [163] M. E. Vallejos, M. D. Zambon, M. C. Area, A. A. da Silva Curvelo, Low liquid-solid ratio (LSR) hot water pretreatment of sugarcane bagasse, *Green Chem.* 14 (2012) 1982–1989.
- [164] J. Söderström, L. Pilcher, M. Galbe, G. Zacchi, Two-step steam pretreatment of softwood with SO<sub>2</sub> impregnation for ethanol production, *Applied Biochemistry and Biotechnology* 98 (2002) 5–21.
- [165] Y. Sun, J. Cheng, Hydrolysis of lignocellulosic materials for ethanol production: a review, *Bioresource Technology* 83 (1) (2002) 1–11.
- [166] E. Gnansounou, A. Dauriat, Techno-economic analysis of lignocellulosic ethanol: A review, *Bioresource Technology* 101 (13) (2010) 4980–4991.
- [167] S. Prasad, A. Singh, H. Joshi, Ethanol as an alternative fuel from agricultural, industrial and urban residues, *Resources, Conservation and Recycling* 50 (1) (2007) 1–39.
- [168] V. Agbor, N. Cicek, R. Sparling, A. Berlin, D. Levin, et al., Biomass pretreatment: fundamentals toward application., *Biotechnology Advances* 29 (6) (2011) 675–685.
- [169] Y. Sun, J. Cheng, Dilute acid pretreatment of rye straw and bermudagrass for ethanol production, *Bioresource Technology* 96 (14) (2005) 1599–1606.
- [170] G. J. de Moraes Rocha, C. Martin, I. B. Soares, A. M. S. Maior, H. M. Baudel, C. A. M. de Abreu, Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production, *Biomass and Bioenergy* 35 (1) (2011) 663–670.
- [171] C. Lloyd, T.A. & Wyman, Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids, *Bioresource Technology* 96 (18) (2005) 1967–1977.
- [172] J. Jensen, J. Morinelly, K. Gossen, M. Brodeur-Campbell, D. Shonnard, Effects of dilute acid pretreatment conditions on enzymatic hydrolysis monomer and oligomer sugar yields for aspen, balsam, and switchgrass, *Bioresource Technology* 101 (7) (2010) 2317–2325.
- [173] E. Castro, M. Díaz, C. Cara, E. Ruiz, I. Romero, M. Moya, Dilute acid pretreatment of rapeseed straw for fermentable sugar generation, *Bioresource Technology* 102 (2) (2011) 1270–1276.
- [174] A. Esteghlalian, A. Hashimoto, J. Fenske, M. Penner, Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass, *Bioresource Technology* 59 (2) (1997) 129–136.
- [175] J. Hernández-Salas, M. Villa-Ramírez, J. Veloz-Rendón, K. Rivera-Hernández, R. González-César, M. Plascencia-Espinosa, S. Trejo-Estrada, Comparative hydrolysis and fermentation of sugarcane and agave bagasse, *Bioresource Technology* 100 (3) (2009) 1238–1245.
- [176] P. Laopaiboon, A. Thani, V. Leelavatcharamas, L. Laopaiboon, Acid hydrolysis of sugarcane bagasse for lactic acid production, *Bioresource Technology* 101 (3) (2010) 1036–1043.
- [177] H. Li, N. Kim, M. Jiang, J. Kang, H. Chang, Simultaneous saccharification and fermentation of lignocellulosic residues pretreated with phosphoric acid-acetone for bioethanol production, *Bioresource Technology* 100 (13) (2009) 3245–3251.
- [178] M. Himmel, W. Adney, J. Baker, R. Elander, J. McMillan, R. Nieves, J. Sheehan, S. Thomas, T. Vinzant,

- M. Zhang, Advanced bioethanol production technologies: a perspective 666 (1997) 2–45.
- [179] A. Rodríguez-Chong, A. Ramirez, G. Garrote, M. Vazquez, Hydrolysis of sugar cane bagasse using nitric acid: a kinetic assessment, *Journal of Food Engineering* 61 (2) (2004) 143–152.
- [180] R. Chandra, H. Takeuchi, T. Hasegawa, Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production, *Renewable and Sustainable Energy Reviews* 16 (3) (2012) 1462–1476.
- [181] S. Zhu, Y. Wu, Z. Yu, J. Liao, Y. Zhang, Pretreatment by microwave/alkali of rice straw and its enzymic hydrolysis, *Process Biochemistry* 40 (9) (2005) 3082–3086.
- [182] Z. Hu, Z. Wen, Enhancing enzymatic digestibility of switchgrass by microwave-assisted alkali pretreatment, *Biochemical Engineering Journal* 38 (3) (2008) 369–378.
- [183] C. Cardona, Ó. Sánchez, Fuel ethanol production: Process design trends and integration opportunities, *Bioresource Technology* 98 (12) (2007) 2415–2457.
- [184] M. Holtzapple, J. Jun, G. Ashok, S. Patibandla, B. Dale, The ammonia freeze explosion (AFEX) process, *Applied Biochemistry and Biotechnology* 28 (1) (1991) 59–74.
- [185] H. Murnen, V. Balan, S. Chundawat, B. Bals, L. Sousa, B. Dale, Optimization of ammonia fiber expansion (AFEX) pretreatment and enzymatic hydrolysis of miscanthus x giganteus to fermentable sugars, *Biotechnology Progress* 23 (4) (2007) 846–850.
- [186] H. Alizadeh, F. Teymouri, T. Gilbert, B. Dale, Pretreatment of switchgrass by ammonia fiber explosion (AFEX), *Applied Biochemistry and Biotechnology* 124 (1) (2005) 1133–1141.
- [187] B. Bals, C. Wedding, V. Balan, E. Sendich, B. Dale, Evaluating the impact of ammonia fiber expansion (AFEX) pretreatment conditions on the cost of ethanol production, *Bioresource Technology* 102 (2) (2011) 1277–1283.
- [188] E. Bahcegul, S. Apaydin, N. I. Haykir, E. Tatli, U. Bakir, Different ionic liquids favor different lignocellulosic biomass particle sizes during pretreatment to function efficiently, *Green Chem.* 14 (2012) 1896–1903.
- [189] A. Brandt, M. Ray, T. To, D. Leak, R. Murphy, T. Welton, Ionic liquid pretreatment of lignocellulosic biomass with ionic liquid–water mixtures, *Green Chem.* 13 (9) (2011) 2489–2499.
- [190] T. Doherty, M. Mora-Pale, S. Foley, R. Linhardt, J. Dordick, Ionic liquid solvent properties as predictors of lignocellulose pretreatment efficacy, *Green Chem.* 12 (11) (2010) 1967–1975.
- [191] H. Zhao, G. Baker, J. Cowins, Fast enzymatic saccharification of switchgrass after pretreatment with ionic liquids, *Biotechnology Progress* 26 (1) (2010) 127–133.
- [192] H. Rodríguez, S. Padmanabhan, G. Poon, J. Prausnitz, et al., Addition of ammonia and/or oxygen to an ionic liquid for delignification of miscanthus., *Bioresource Technology* 102 (17) (2011) 7946–7952.
- [193] J. Binder, R. Raines, Simple chemical transformation of lignocellulosic biomass into furans for fuels and chemicals, *Journal of the American Chemical Society* 131 (5) (2009) 1979–1985.
- [194] H. Itoh, M. Wada, Y. Honda, M. Kuwahara, T. Watanabe, Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi, *Journal of Biotechnology* 103 (3) (2003) 273–280.
- [195] X. Zhao, K. Cheng, D. Liu, Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis,

- Applied Microbiology and Biotechnology 82 (5) (2009) 815–827.
- [196] F. Sun, H. Chen, Organosolv pretreatment by crude glycerol from oleochemicals industry for enzymatic hydrolysis of wheat straw, *Bioresource Technology* 99 (13) (2008) 5474–5479.
  - [197] J. Zhu, X. Pan, Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation, *Bioresource Technology* 101 (13) (2010) 4992–5002.
  - [198] J. Kristensen, C. Felby, H. Jørgensen, Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose, *Biotechnology for Biofuels* 2 (1) (2009) 11.
  - [199] A. Petersson, M. Thomsen, H. Hauggaard-Nielsen, A. Thomsen, Potential bioethanol and biogas production using lignocellulosic biomass from winter rye, oilseed rape and faba bean, *Biomass and Bioenergy* 31 (11-12) (2007) 812–819.
  - [200] F. Monlau, A. Barakat, J. Steyer, H. Carrere, Comparison of seven types of thermo-chemical pretreatments on the structural features and anaerobic digestion of sunflower stalks, *Bioresource Technology* 120 (0) (2012) 241 – 247.
  - [201] J. Zhu, C. Wan, Y. Li, Enhanced solid-state anaerobic digestion of corn stover by alkaline pretreatment, *Bioresource Technology* 101 (19) (2010) 7523–7528.
  - [202] H. Gavala, I. Angelidaki, B. Ahring, *Kinetics and modeling of anaerobic digestion process*, Springer, 2003.
  - [203] V. Vavilin, B. Fernandez, J. Palatsi, X. Flotats, Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview, *Waste Management* 28 (6) (2008) 939–951.
  - [204] P. Colberg, Anaerobic microbial degradation of cellulose, lignin, oligolignols, and monoaromatic lignin derivatives, *Biology of Anaerobic Microorganisms* (1988) 333–372.
  - [205] B. Ahring, M. Sandberg, I. Angelidaki, Volatile fatty acids as indicators of process imbalance in anaerobic digestors, *Applied Microbiology and Biotechnology* 43 (1995) 559–565.
  - [206] I. Angelidaki, L. Ellegaard, B. Ahring, A comprehensive model of anaerobic bioconversion of complex substrates to biogas, *Biotechnology and Bioengineering* 63 (3) (1999) 363–372.
  - [207] K. Hansen, I. Angelidaki, B. Ahring, Anaerobic digestion of swine manure: inhibition by ammonia, *Water Research* 32 (1) (1998) 5–12.
  - [208] R. Biswas, Biomethanation of red algae from the eutrophied baltic sea, Master of Science thesis, Department of Water and Environmental Studies, Linköping University, Sweden (2009) 1–65.  
URL <http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-51338>
  - [209] G. Lissens, A. Thomsen, L. De Baere, W. Verstraete, B. Ahring, Thermal wet oxidation improves anaerobic biodegradability of raw and digested biowaste, *Environmental Science & Technology* 38 (12) (2004) 3418–3424.
  - [210] S. Menardo, P. Balsari, E. Dinuccio, F. Gioelli, Thermal pre-treatment of solid fraction from mechanically-separated raw and digested slurry to increase methane yield, *Bioresource Technology* 102 (2) (2011) 2026–2032.
  - [211] G. Wang, Biogas production from energy crops and agriculture residues, Ph.D. thesis, Technical University of Denmark, Risø National Laboratory for Sustainable Energy, Biosystems Division (2010).
  - [212] P. Strong, D. Gapes, Thermal and thermo-chemical pre-treatment of four waste residues and the effect on

- acetic acid production and methane synthesis, *Waste Management* 32 (9) (2012) 1669 – 1677.
- [213] A. Bauer, P. Bösch, A. Friedl, T. Amon, Analysis of methane potentials of steam-exploded wheat straw and estimation of energy yields of combined ethanol and methane production, *Journal of Biotechnology* 142 (1) (2009) 50–55.

***C. Research paper I***  
***(Paper II)***

---

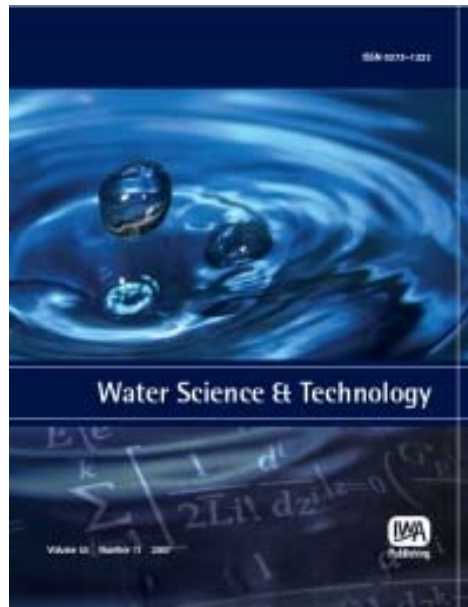
***Improving biogas yields using an innovative concept  
for conversion of the fiber fraction of manure***

RAJIB BISWAS, B. K. AHRING AND H. UELLEND AHL

*(Published in Water Science & Technology (2012), 66 (8), 1751–1758.)*



**Provided for non-commercial research and educational use only.  
Not for reproduction or distribution or commercial use.**



This article was originally published by IWA Publishing. IWA Publishing recognizes the retention of the right by the author(s) to photocopy or make single electronic copies of the paper for their own personal use, including for their own classroom use, or the personal use of colleagues, provided the copies are not offered for sale and are not distributed in a systematic way outside of their employing institution.

Please note that you are not permitted to post the IWA Publishing PDF version of your paper on your own website or your institution's website or repository.

Please direct any queries regarding use or permissions to [wst@iwap.co.uk](mailto:wst@iwap.co.uk)

## Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure

Rajib Biswas, B. K. Ahring and H. Uellendahl

### ABSTRACT

The potential of a new concept to enable economically feasible operation of manure-based biogas plants was investigated at laboratory scale. Wet explosion (WEx) was applied to the residual manure fibers separated after the anaerobic digestion process for enhancing the biogas yield before reintroducing the fiber fraction into the biogas reactor. The increase in methane yield of the digested manure fibers was investigated by applying the WEx treatment under five different process conditions. The WEx treatment at 180 °C and a treatment time of 10 min without addition of oxygen was found to be optimal, resulting in 136% increase in methane yield compared with the untreated digested manure fibers in batch experiments. In a continuous mesophilic reactor process the addition of WEx-treated digested fibers in co-digestion with filtered manure did not show any signs of process inhibition, and the overall methane yield was on average 75% higher than in a control reactor with addition of non-treated digested fibers.

**Key words** | anaerobic degradation, digested manure fibers, methane yield, wet explosion treatment

Rajib Biswas

B. K. Ahring

H. Uellendahl (corresponding author)

Section for Sustainable Biotechnology,

Aalborg University Copenhagen,

Lautrupvang 15,

2750 Ballerup,

Denmark

E-mail: [hu@bio.aau.dk](mailto:hu@bio.aau.dk)

B. K. Ahring

Center for Bioproducts and Bioenergy,

Washington State University,

2710 Crimson Way,

Richland,

WA 99354,

USA

### INTRODUCTION

Biogas produced from manure, organic waste and plant biomass is becoming increasingly attractive in terms of reducing greenhouse gas emission, nutrient recovery and well as renewable energy alternatives to fossil fuels. It was shown in a recent life cycle assessment that biogas production from manure has the highest reduction effect on greenhouse gas emissions compared with other biofuels production processes (Thyø & Wenzel 2007). Despite the environmental benefits, the economical operation of centralized biogas plants based on manure alone is difficult due to a low methane yield per volume unit of manure (Gerin *et al.* 2008). Thus manure-based biogas plants are currently depending on the co-digestion of industrial waste with a high methane yield, typically originating from the food industry. While for example 40 million tons of manure produced annually in Denmark represents a huge biogas potential, only 5% of this amount is currently treated in biogas plants, and the implementation of centralized biogas plants in Denmark has stagnated throughout the last 10 years due to the fact that the operation based on manure alone has not been viable and the availability of industrial organic waste is limited (Jensen *et al.* 2009). The Danish governmental program 'Green Growth' targets an

exploitation of up to 40% of manure in 2020 (Danish Government 2009), corresponding to an increase in biogas plant capacity by 8-fold, equivalent to more than 70 new centralized biogas plants of the largest scale. As manure will be the main substrate for these future biogas plants it is a prerequisite to achieve an economically feasible operation of manure-based biogas plants in order to see this program come into full implementation. For economic operation of a biogas plant, biogas yields of more than 30 m<sup>3</sup> per m<sup>3</sup> feed are needed to compensate for the transportation costs (Uellendahl *et al.* 2007). The biogas yield of manure in conventional biogas plants is often lower as the organic matter content in manure is typically less than 10%, of which 60–80% is fiber material, which leads to methane yields of only 30–50% of the methane potential (Hartmann *et al.* 2000; Christensen *et al.* 2007; Boe & Angelidaki 2009). Intensive research has been carried out for improving the biogas yield of manure and sludge by implementing a wide range of biological, chemical, mechanical, and thermal pretreatment methods (Angelidaki & Ahring 2000; Hartmann *et al.* 2000; Carrère *et al.* 2010), in co-digestion with organic waste (Angelidaki & Ellegaard 2003), in combination with solid–liquid separation of manure

(Mladenovska *et al.* 2006; Christensen *et al.* 2007; Møller *et al.* 2007; Kaparaju & Rintala 2008), and in different digester configurations (Boe & Angelidaki 2009; Kaparaju *et al.* 2009). Comparing the different treatment methods for increasing the biofuel yield from lignocellulosic biomass, it was found that thermo-chemical treatment was the most suitable for the treatment of lignocellulosic biomass for subsequent conversion into biogas (Angelidaki & Ahring 2000; Lissens *et al.* 2004; Uellendahl *et al.* 2007). Thermal hydrolysis has proven commercial viability for enhancing biogas production of sludge and household waste through the implementation of a number of large-scale plants worldwide by the company Cambi (Elliott & Mahmood 2007).

Wet explosion (WEx), a steam explosion process with or without addition of oxygen, has previously shown a high potential for the destruction of the lignocellulosic structure of biomass, in order to enable the hydrolysis for subsequent ethanol fermentation (Klinke *et al.* 2002; Lissens *et al.* 2004; Sørensen *et al.* 2008). Generally, WEx includes both physical disruption and a partly chemical degradation of the biomass (Sørensen *et al.* 2008). The WEx treatment equipment, patented by the Danish company Biogasol ApS, can handle material with up to 30% dry matter, and results in high sugar yields, which can subsequently be converted into ethanol or methane (Christensen *et al.* 2007; Ahring & Langvad 2008). Previous studies revealed that the effect of the WEx treatment is correlated to the content of lignin in lignocellulosic fiber material (Uellendahl *et al.* 2007). As a consequence, the combination of the WEx treatment

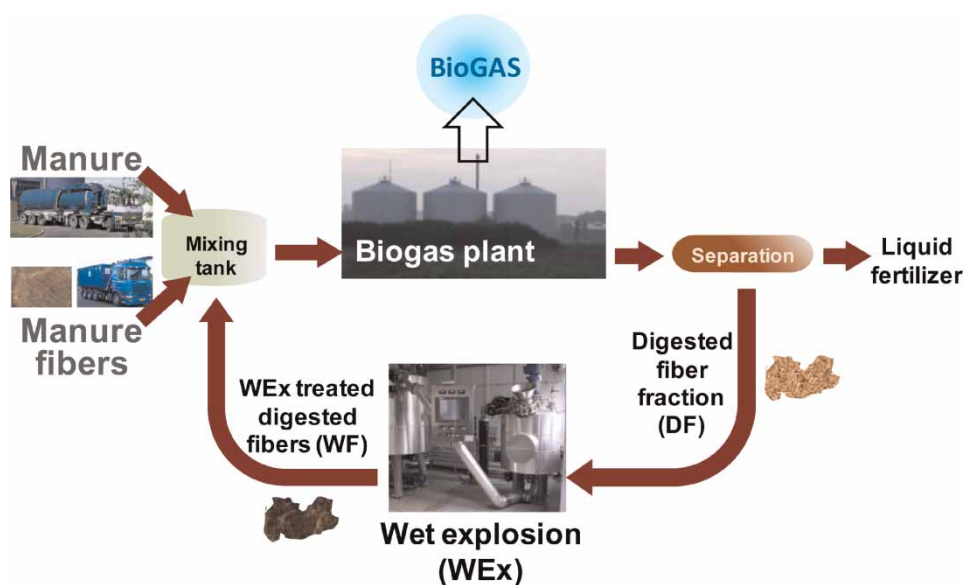
with biogas production from manure was evaluated to be most beneficial when applying the treatment to manure fibers separated from the effluent of a biogas reactor after digestion (Figure 1).

In the present research project called FiberMaxBiogas the potential of the new concept of combining the recirculation of the digested fiber fraction with the WEx treatment for increasing the biogas yield of manure is investigated. In the following we will present the first overall results from batch tests for screening of the increase of the biogas yield of the digested fiber fraction after WEx treatment and the long-term performance of adding the WEx-treated digested fibers in co-digestion with manure in laboratory-scale reactors.

## MATERIAL AND METHODS

### Digested fiber fraction

The effect of the WEx treatment was tested on digested fibers separated from the effluent of one of the biogas reactors of the Biokraft A/S centralized biogas plant on Bornholm, Denmark. For separation of the digested fiber fraction an industrial-scale decanter centrifuge was used. The Biokraft A/S biogas plant is operated under mesophilic conditions (38 °C) with a hydraulic retention time (HRT) of 20 days, treating manure (>90% vol.), co-digested with agricultural residues (<5% vol.) and industrial waste (<5% vol.) from food-processing industries.



**Figure 1** | Overview of the new manure-to-biogas concept with WEx treatment of the digested fiber fraction separated from the effluent.

## Filtered manure

The digested fiber fraction was added to the laboratory-scale reactors in co-digestion with filtered cow manure (FCM). FCM was obtained from cow manure delivered to the biogas plant of Biokraft A/S filtered through 10 mm sieves in order to avoid clogging of the influent tube. Filtered mixed manure (FMM) was used for the co-digestion of the digested fiber fraction from day 138 to 180. FMM was obtained from a mixture of manure from cows, pigs and poultry in a ratio of 70:25:5 on a total solids (TS) basis.

## Wet explosion treatment

Wet explosion treatment was performed in a laboratory-scale 3.5 L batch reactor with a maximum active volume of 2.0 L, provided by Biogasol A/S. The reactor is equipped with continuous stirring (990 rpm) a gas and liquid dosage system for supply of additives ( $\text{H}_2\text{O}_2$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{O}_2$ ,  $\text{Na}_2\text{CO}_3$  etc.), and a flush valve for sudden pressure release into a 25 L subsequent flash tank. The reactor is heated by an external oil heater. The denoted process temperature was the temperature measured at the reactor top.

Digested fibers were treated in 1 kg batches, adding 400 g of tap water to 600 g of fiber material for achieving a TS concentration of 12% inside the reactor. After the denoted treatment time in the reactor, the biomass was flushed into the flush tank. The different treatment conditions are displayed in Table 1. Heating times to reach the start temperature varied between 7 and 15 min due to the different final temperature.

## Batch experiments

The methane yields of treated and untreated digested fibers were determined in laboratory-scale anaerobic batch tests using 117 mL vials under mesophilic condition ( $38 \pm 0.5^\circ\text{C}$ ).

Inoculum for the batch experiments was supplied from one of Biokraft's biogas reactors and stored at  $4^\circ\text{C}$ . Before batch set-up, the inoculum was pre-incubated at  $38^\circ\text{C}$  for one week.

Two different inoculum-to-substrate ratios (ISRs) were tested, i.e., 1.0 g volatile solids (VS)/vial (ISR 1) and 0.5 g-VS/vial (ISR 2) of the substrate were added to vials with 25 mL inoculum. After filling with the respective biomass and inoculum, batch vials were flushed with  $\text{N}_2/\text{CO}_2$  (80%/20%) prior to closing air tight with rubber stoppers and aluminum crimps. Experimental set-up was performed in triplicate and a triplicate of vials filled with 25 mL inoculum and water instead of substrate was used as control. The vials were incubated until no significant further biogas production was detected (48 days). The methane yield of the treated and untreated digested fibers was determined by measuring the methane concentration in the headspace using gas chromatography (GC) (SRI-GC-310) and calculated according to Equation (1). Overpressure in the vials was released whenever necessary and the methane concentration in the headspace was determined before and after the gas release for calculation of the cumulative methane yield. Methane production in the controls filled with inoculum only was subtracted to calculate the methane yield from the added substrate ( $\text{mL/g-VS}_{\text{added}}$ ). A gas mixture of  $\text{CH}_4/\text{N}_2$  (30%/70%) was used as standard gas mixture for gas GC.

$$\text{CH}_4\text{yield}_S = (\text{CH}_4\%_S \cdot V_{\text{headspace},S} - \text{CH}_4\%_C \cdot V_{\text{headspace},C}) / \text{g-VS}_{\text{added},S} \quad (1)$$

where index S: added substrate; index C: control vials (without substrate).

## CSTR experiments

Two 5 L stainless steel continuous stirred tank reactors (CSTRs) with a working volume of 3 L were operated with a HRT of 20 days. The reactor temperature was maintained at  $38 \pm 0.5^\circ\text{C}$  by circulating hot water in the heating jacket using a water bath. In order to evaluate the biogas process of WEx-treated digested fibers, CSTR experiments were performed by feeding WEx-treated digested fibers (WF) in a test reactor (R1) and non-treated digested fibers (DF) in a control reactor (R2). The fiber fraction was in both reactors co-digested together with FCM. The WF were pretreated at  $180^\circ\text{C}$  for 10 min. Both reactors were fed (150 mL) twice a day using peristaltic pumps (Watson-Marlow 610 series). The produced biogas was registered using volumetric gas

**Table 1** | WEx conditions tested for the treatment of digested manure fibers (DF)

Batch	Treatment time (min)	Temperature ( $^\circ\text{C}$ )	Pressure (bar)	Addition of $\text{O}_2$ (bar)
145-10	10	145	2.2	–
165-10	10	165	3.3	–
165-20	20	165	7.4	–
165-10- $\text{O}_2$	10	165	12.4	6
180-10	10	180	9.9	–

meters, logging the gas production automatically in 10 mL intervals. Reactors were stirred for 5 min 10 times a day. The performance of the reactors was monitored on the basis of methane yield, volatile fatty acids (VFAs) concentration and pH.

During start-up, both reactors R1 and R2 were filled with 3 L of inoculum, originating from one of Biokraft's biogas reactors. The feeding was started with FCM alone (days 0–54) with an organic loading rate (OLR) of  $2.5 \text{ g} \cdot \text{VS}/(\text{L} \cdot \text{d})$  and a HRT of 20 days. On day 55, co-digestion of the DF with FCM was initiated in R1 and R2 at a feed ratio of 1:1 (w/w, % VS basis) with an increased OLR of  $3.5 \pm 0.5 \text{ g-VS}/(\text{L} \cdot \text{d})$  between days 55 and 76. After reaching the steady-state in both reactors on day 77, WF was gradually introduced in R1, replacing the same amount of VS of the DF in feed until only WF was used for co-digestion with FCM in feed of R1 (day 101). The feeding mixture in R2 was kept unchanged as on day 76 until day 214 when the experiments were terminated. From day 138 to day 180 FMM was added instead of FCM in both reactors.

## Analytical methods

Analyses of TS, total suspended solids (TSS), total dissolved solids (TDS), volatile suspended solids (VSS), VS, and chemical oxygen demand (COD) were carried out for both raw and pretreated material. COD was determined in Hach Lange cuvette tests according to the company's method LCK 914. TS, TSS, VSS, and VS were analyzed in accordance with standard methods (American Public Health Association *et al.* 2005). Samples from both reactors were taken for measuring pH and VFAs two to three times per week.  $125 \mu\text{L}$  of 17%  $\text{H}_3\text{PO}_4$  was added in 1 mL of sample in a 2 mL Eppendorf tube and centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into VFA vials for analysis in a gas chromatograph

PerkinElmer Clarus 400 series, equipped with flame ionization detector (FID) and a Hewlett Packard FFAP capillary column,  $30 \text{ m} \times 0.53 \text{ mm}$  I.D., film thickness  $1.0 \mu\text{m}$ , using nitrogen as a carrier gas. The oven temperature was programmed from  $115^\circ\text{C}$  (hold for 3 min) to  $125^\circ\text{C}$  at a rate of  $5^\circ\text{C}/\text{min}$  and then increasing  $45^\circ\text{C}/\text{min}$  to  $230^\circ\text{C}$  and held at final temperature for 2 min. Nitrogen was used as a carrier gas at  $18 \text{ mL}/\text{min}$  and the injector port and detector temperature were  $175^\circ\text{C}$  and  $200^\circ\text{C}$  respectively. Methane content ( $\text{CH}_4$ ) in produced biogas for both batch and CSTR experiments was measured 2–3 times per week using GC (SRI-GC-310), SRI Instruments, USA, equipped with thermal conductivity detector and a packed column (Porapak-Q,  $6 \text{ ft} \times 2.1 \text{ mm}$  I.D.), where nitrogen was also used as a carrier gas. The pH was measured using an InoLab® pH 727 meter (WTW Inc.), with precise measurement values (0.001 pH).

## RESULTS AND DISCUSSION

### Effect of wet explosion on substrate characteristics

The characteristics of DF and WEx-treated DF under five different treatment conditions (145-10, 165-10, 165-20, 165-10- $\text{O}_2$ , and 180-10) are displayed in Table 2. The TS and VS of the digested manure fibers were 20.0 and 14.8%, respectively, with a COD/VS ratio of 1.5. Generally, the COD/VS ratio did not alter significantly during WEx treatment (145-10, 165-10, 165-20 and 165-10- $\text{O}_2$ ). However, a significant higher COD/VS ratio of 1.7 was found for the WEx-treated fibers 180-10, where the treatment was performed at  $180^\circ\text{C}$  and 10 min treatment time. This may be explained by the fact that due to the higher temperature a higher amount of lignin was broken into lower molecular compounds like phenols with a higher COD/VS ratio.

**Table 2** | Characteristics of digested manure fibers before and after WEx treatment under five different conditions

Batch	TS (g/L)	VS (g/L)	TSS % of TS	TDS % of TS	VSS (g/L)	COD (g/L)	COD/VS	pH
DF	199.5 (2.4)	147.6 (1.9)	89.4 (0.7)	10.6 (0.7)	136.1 (4.6)	218.5 (15.7)	1.48	8.31
145-10	120.1 (2.6)	89.5 (2.2)	86.5 (2.1)	13.5 (2.1)	78.3 (2.1)	135.2 (0.0)	1.51	9.04
165-10	121.0 (2.9)	89.9 (2.5)	82.8 (2.3)	17.2 (2.3)	75.7 (1.0)	131.3 (0.0)	1.46	8.79
165-20	118.6 (2.4)	89.6 (2.2)	83.9 (0.5)	16.1 (0.5)	74.3 (1.6)	130.8 (1.3)	1.46	8.84
165-10- $\text{O}_2$	109.7 (0.2)	80.7 (0.6)	77.9 (0.0)	22.1 (0.0)	53.5 (0.6)	123.2 (9.0)	1.53	7.60
180-10	120.5 (1.9)	89.9 (1.7)	80.0 (0.6)	20.0 (0.6)	71.9 (0.7)	152.7 (5.5)	1.70	8.80

Samples were diluted before WEx treatment (3:2); values in brackets are standard deviation.



The relatively high pH of the digested fibers even increased for all WEx treatment conditions except for batch 165-10-O<sub>2</sub> where O<sub>2</sub> was added. Furthermore, TS, and VS after the treatment 165-10-O<sub>2</sub> were lower than for the untreated material, obviously due to a higher conversion of the material to CO<sub>2</sub> by addition of O<sub>2</sub>. The amount of TDS in the pretreated materials increased under all five conditions.

### Change of methane yield by wet explosion

The course of methane production and the final methane yields during the batch digestion of DF and WEx-treated DF under the five tested WEx conditions are displayed in Figure 2. Generally, increasing the treatment temperature resulted in higher methane yields (Figure 2(a)). The highest methane yield (224 mL/g-VS) was found for the digested fibers treated at 165 °C under the addition of oxygen at a batch loading of 0.5 g-VS/vial, ISR 2 (Figure 2(b)).

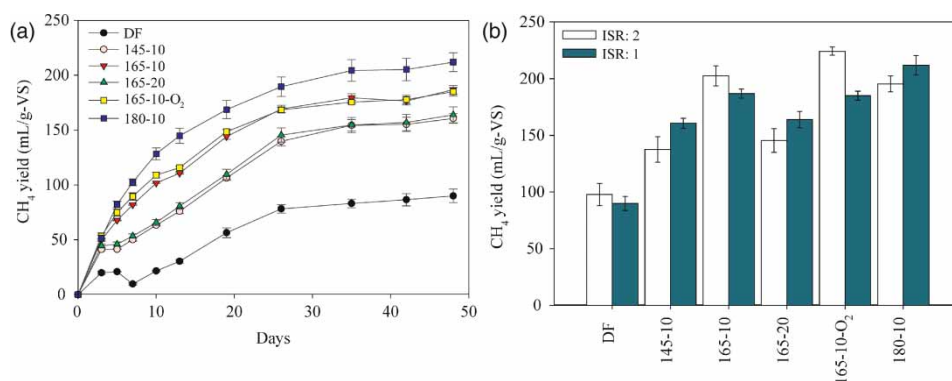
At a higher load the final methane yield of the material treated with addition of O<sub>2</sub> was, however, significantly lower. This indicated an inhibiting effect from the WEx-treated material with oxygen that counteracted the increase in degradability. For all other treatment conditions the increase in loading of the batch vial had only a minor effect, indicating no or only low production of inhibiting compounds during WEx treatment. Without addition of oxygen, the WEx treatment at 180 °C for 10 min resulted in the highest increase of the methane yield of 136%.

### CSTR Experiments

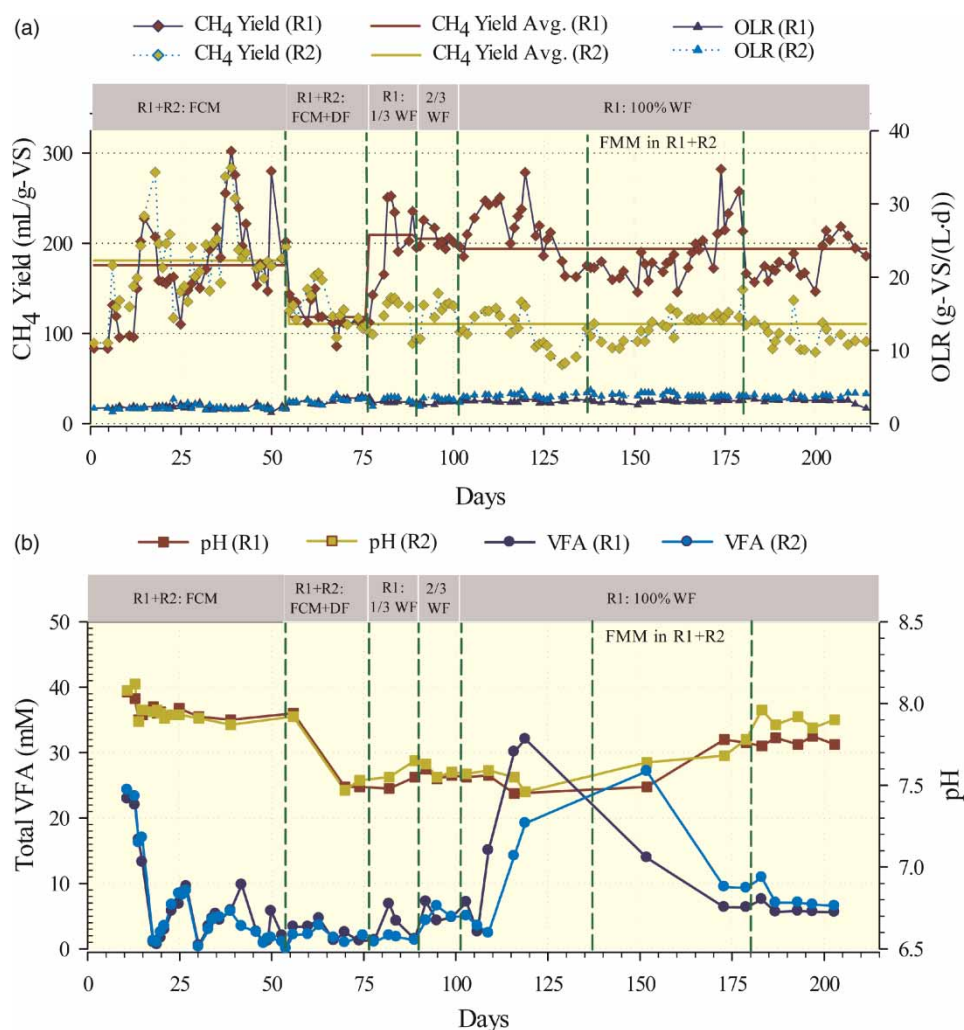
The performance of the test reactor R1 and control reactor R2 was monitored for 214 days by methane yield, VFA

concentration, and pH (Figure 3). In the initial start-up, both reactors were fed with FCM alone for 54 days. From day 55 untreated digested manure fibers were added to the feed of both reactors with the filtered manure. While maintaining the OLR at  $3.5 \pm 0.5$  g-VS/(L · d) the methane yield per gram organic matter decreased in both reactors significantly from around 180 mL/g-VS<sub>added</sub> to 118 and 111 mL/g-VS<sub>added</sub>, in R1 and R2, respectively, due to the higher content of organic matter with a low degradability. After steady-state conditions were established in both reactors, the untreated fibers DF in the reactor feed of R1 were from day 77 gradually replaced by WEx-treated fibers WF, replacing 1/3 of the DF from day 76, 2/3 from day 90 and feeding 100% WF in co-digestion with FCM from day 101. The average methane yield in R1 increased in the following days gradually and reached on average 194 mL/g-VS<sub>added</sub> (days 101 to 214) when feeding WF compared with 111 mL/g-VS<sub>added</sub> in the control reactor R2 with untreated DF (Figure 3(a)). Both reactors show a decrease in methane yield from day 121 to 140 due to a process disturbance after blockage in the influent tube of both reactors, which made cleaning of the reactors necessary. The performance of both reactors recovered, however, during the period from day 137 to 180, when the fiber fractions were co-digested in both reactors with FMM. Despite these fluctuations the methane yield in R1 remained significantly higher than in R2 for all times.

During start-up of both reactors the VFA concentration in both reactors exhibited very similar patterns with a rise above 20 mM and subsequent decrease to values lower than 10 mM (Figure 3(b)). This indicated very similar performance of both reactors with an adaptation phase during start-up. Also when introducing WEx-treated fibers WF in R1 on day 76 the VFA concentrations remain



**Figure 2** | Accumulated methane yield in batch experiments of DF and WEx-treated DF under five difference WEx conditions (145-10, 165-10, 165-20, 165-10-O<sub>2</sub>, and 180-10). (a) Methane yields for a batch load of 1.0 g-VS/vial (ISR 1) and (b) final methane yields after 48 days for the two different batch loads (ISR 2 and ISR 1). Error bars indicate the standard deviation of three replications.



**Figure 3** | Methane yield, OLR (a), VFA and pH (b) in reactor R1 and R2 in the different experimental phases. Phase 1 (until day 54): start-up of both reactors with FCM. Phase 2 (day 55–76): addition of digested fibers in R1 and R2. Phase 3–5 (day 77–214): change of feed in R1 to WF.

generally very similar in both reactors. In the period from day 107 to 119 a significant increase of VFA up to 19 mM in R2 and 32 mM in R1 was observed. Although the origin of this increase remained unclear the higher increase in R1 may indicate that the performance of reactor R1 was more sensitive when only WEx-treated fibers were added. On day 152 an increase of the VFA to 27 mM in R2 was caused by a process disturbance after blockage in the effluent tube. In the long run, however, and despite the change of FCM to FMM from day 137 to 180, the VFA concentration remained low in both R1 and R2. Furthermore, the generally very stable process performance of both reactors can be seen by the pH values of the reactors, which remained  $7.6 \pm 0.2$  throughout the whole operation period.

## Economy

The treatment of the digested fiber fraction in the new treatment concept offers two economical benefits: the biogas yield per ton of manure feedstock increases and the costs for treating only the separated digested fiber fraction are significantly lower than for the pretreatment of the whole reactor feed. In the case of Biokraft's biogas plant roughly 100 kg of separated digested fibers are leaving the reactor per ton of input. Consequently, the volume to be treated is only 10% compared with pretreatment of the whole input, reducing the operational costs accordingly. Through mass balance based on Biokraft's production data (Biokraft 2012) and the results found for increasing the biogas yield by WEx treatment of the digested fibers, it shows that the

methane yield per ton of feed can be increased from 23 to 28 m<sup>3</sup> by the recirculation and to 33 m<sup>3</sup> when the biodegradability of the fiber fraction is enhanced from 40 to 75%. This could make the new concept economically viable. More detailed values for investment and operational costs for the recirculation and WEx treatment will be available from a large-scale test.

## CONCLUSION

The testing of a new concept for increasing the biogas yield of manure by combination of anaerobic digestion with wet explosion of the digested fiber fractions showed in both batch and reactor experiments that the methane yield of the fiber fraction can be significantly enhanced. Testing the WEx treatment under different conditions revealed optimum conditions at a temperature of 180 °C and a treatment time of 10 min without addition of oxygen, resulting in a 136% higher methane yield as compared with the untreated digested fibers in batch experiments. The continuous feeding of WEx-treated fibers in co-digestion with filtered manure revealed on average a 75% higher total yield. The batch experiments indicate that the addition of oxygen during the WEx treatment may lead to inhibiting compounds. The reactor experiments with digested fibers treated at 180 °C for 10 min revealed no significant signs of inhibition after a short adaptation phase when introducing WEx-treated fibers in co-digestion with manure. The proof of this concept in large scale will be followed by demo-scale testing of the concept at Biokraft's biogas plant on Bornholm.

## ACKNOWLEDGEMENT

The FiberMaxBiogas project is supported by a grant from energinet.dk, the Danish supplier of the electricity and gas grid.

## REFERENCES

- Ahring, B. K. & Langvad, N. 2008 Sustainable low cost production of lignocellulosic bioethanol – 'The carbon slaughterhouse'. A process concept developed by BioGasol. *International Sugar Journal* **110** (1311), 184–190.
- American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF) 2005 *Standard Methods for the Examination of Water and Wastewater*. (A. E. Greenberg, L. S. Clesceri & R. R. Trussel, ed.) 21st edition. APHA, Washington, DC, USA.
- Angelidaki, I. & Ahring, B. K. 2000 Methods for increasing the biogas potential from the recalcitrant organic matter contained in manure. *Water Science and Technology* **41** (3), 189–194.
- Angelidaki, I. & Ellegaard, L. 2003 Codigestion of manure and organic wastes in centralized biogas plants: status and future trends. *Applied Biochemistry and Biotechnology* **109**, 95–105.
- Biokraft 2012 Biokraft produktionsdata 2011–2012. Available from: <http://biokraft.dk/produktionsdata.asp> (accessed on April 16, 2012).
- Boe, K. & Angelidaki, I. 2009 Serial CSTR digester configuration for improving biogas production from manure. *Water Research* **43**, 166–172.
- Carrère, H., Dumas, C., Battimelli, A., Batstone, D. J., Delgenès, J. P., Steyer, J. P. & Ferrer, I. 2010 Pretreatment methods to improve sludge anaerobic degradability: a review. *Journal of Hazardous Compounds* **183**, 1–15.
- Christensen, J., Hjort-Gregersen, K., Uellendahl, H., Ahring, B. K., Lau Baggesen, D., Stockmarr, A., Møller, H. B. & Birkmose, T. 2007 Fremtidens biogasfællesanlæg – nye anlægskoncepter og økonomisk potentiale. Report no. 188, Fødevareøkonomisk Institute.
- Danish Government 2009 Grøn Vækst. Økonomi- og Erhvervsministeriet, Copenhagen, Denmark.
- Elliott, A. & Mahmood, T. 2007 Pretreatment technologies for advancing anaerobic digestion of pulp and paper biotreatment residues. *Water Research* **41**, 4273–4286.
- Gerin, P. A., Vliegen, F. & Jossart, J. M. 2008 Energy and CO<sub>2</sub> balance of maize and grass as energy crops for anaerobic digestion. *Bioresource Technology* **99**, 2620–2627.
- Hartmann, H., Angelidaki, I. & Ahring, B. K. 2000 Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration. *Water Science and Technology* **41** (3), 145–153.
- Jensen, N. E. H., Thomassen, H., Jensen, J. B., Jakobsen, K. R. & Jacobsen, S. H. 2009 Energy 2009: Annual report on Danish energy research programmes. ISSN number: Printed version: 1903–9565, Digital version: 1902–8318. Available from: <http://en.fi.dk/publications/2009/energy09/energy09-UK-low.pdf> (accessed on June 8, 2011).
- Kaparaju, P. L. N. & Rintala, J. A. 2008 Effects of solid-liquid separation on recovering residual methane and nitrogen from digested dairy cow manure. *Bioresource Technology* **99**, 120–127.
- Kaparaju, P., Ellegaard, L. & Angelidaki, I. 2009 Optimisation of biogas production from manure through serial digestion: lab-scale and pilot scale studies. *Bioresource Technology* **100**, 701–709.
- Klinke, H. B., Ahring, B. K., Schmidt, A. S. & Thomsen, A. B. 2002 Characterization of degradation products from alkaline wet oxidation of wheat straw. *Bioresource Technology* **82** (1), 15–26.
- Lissens, G., Thomsen, A. B., de Baere, L., Verstraete, W. & Ahring, B. K. 2004 Thermal wet oxidation improves anaerobic biodegradability of raw and digested biowaste. *Environmental Science and Technology* **38**, 3418–3424.
- Mladenovska, Z., Hartmann, H., Kvist, T., Sales-Cruz, M., Gani, R. & Ahring, B. K. 2006 Thermal pretreatment of the solid



- fraction of manure: impact on the biogas reactor performance and microbial community. *Water Science and Technology* **53** (8), 59–67.
- Møller, H. B., Nielsen, A. M., Nakakubo, R. & Olsen, H. J. 2007 Process performance of biogas digesters incorporating pre-separated manure. *Livestock Science* **112**, 217–223.
- Sørensen, A., Teller, P. J., Hilstrøm, T. & Ahring, B. K. 2008 Hydrolysis of *Miscanthus* for bioethanol production using dilute acid presoaking combined with wet explosion pretreatment and enzymatic treatment. *Bioresource Technology* **99** (14), 6602–6607.
- Thyø, K. A. & Wenzel, H. 2007 *Life Cycle Assessment of Biogas from Maize Silage and from Manure*, Institute for Product Development, Lyngby, Denmark, 2007, Available from: [http://www.xergi.com/images/stories/pdf/life\\_cycle\\_assessment\\_report.pdf](http://www.xergi.com/images/stories/pdf/life_cycle_assessment_report.pdf) (accessed on June 8, 2011).
- Uellendahl, H., Mladenovska, Z. & Ahring, B. K. 2007 Wet oxidation of crude manure and manure fibers: Substrate characteristics influencing the pretreatment efficiency for increasing the biogas yield of manure. In *Proceedings 11th World Congress on Anaerobic Digestion*, 23–27 September, 2007, Brisbane, Australia.

First received 16 February 2012; accepted in revised form 20 April 2012

***D. Research paper II***  
***(Paper III)***

---

*Wet explosion pretreatment of sugarcane bagasse for  
enhanced enzymatic hydrolysis*

RAJIB BISWAS, H. UELLEND AHL AND B. K. AHRING

*(Intended for submission to Bioresource Technology)*

# Wet explosion pretreatment of sugarcane bagasse for enhanced enzymatic hydrolysis

Rajib Biswas<sup>a,b</sup>, H. Uellendahl<sup>a</sup>, B.K. Ahring<sup>a,b</sup>

<sup>a</sup>*Section for Sustainable Biotechnology, Aalborg University Copenhagen, A C Meyers Vænge 15, 2450 Copenhagen SV, Denmark*

<sup>b</sup>*Center for Bioproducts and Bioenergy, Washington State University, 2710 Crimson Way, Richland, WA 99354, USA (Email: bka@tricity.wsu.edu)*

---

## Abstract

In this study we tested wet explosion pretreatment with sugarcane bagasse with the aim of obtaining the highest possible sugar yield after pretreatment. The temperatures used were 155, 170, 185 and 200 °C with addition of 6 bar oxygen pressure. Similar pretreatments at the target temperatures without oxygen was further done as a control. The concentration of sugars (glucose, xylose, arabinose) released in the liquid phase as well as degradation products such as acetate, HMF, furfural were determined along with an characterization of the composition of the solid fractions after pretreatment. Two loadings of enzyme mixtures (12.4 and 22.0 mg enzyme protein (EP)/g cellulose) were tested on washed solid cake. Enzymatic efficiency after pretreatment was significantly improved using 22.0 mg EP/g cellulose compared to the lower dose. A pretreatment temperature of 185 °C with oxygen yielded the highest sugar concentration in the liquid fraction (34.0 g/L) leaving 59.2% glucan in the solid fraction resulting in a glucose concentration of 573.9±4.5 g/Kg TS and a glucose yield of 87.4±0.7% of the theoretical maximum value. Although the washed solid sample pretreated at 200 °C with oxygen exhibited enhanced enzymatic efficiency (94.8±0.5%), the condition was not favorable due to lower xylose recovery and formation of the degradation products such as acetate, furfural and HMF of 7.6, 3.3 and 1.0 g/L, respectively, which are known to inhibit microbial growth.

## Keywords:

Wet explosion pretreatment, sugarcane bagasse, enzymatic hydrolysis, inhibitor formation, sugar monomers, bioenergy production.

---

## 1. Introduction

Due to the uncertainty over fossil fuel reserves and rising oil prices, combined with increasing concerns over global climate change, the needs for alternative transportation fuels and non-fossil carbon based materials are evident. Thus, extensive research activities have been conducted to develop processes for biorefineries. Biofuels such as biodiesel and first generation ethanol (sugar- or starch-based) are considered more technologically matured than that of lignocellulosic feedstocks such as agricultural, municipal and farm wastes, marine biomass and energy crops such as miscanthus and switchgrass. Bioproducts and biofuels based on lignocellulosic feedstocks are regarded as a more desirable path due to both sustainability, technically

November 5, 2012

viable, environmental and socio-economic standpoints (Ahring et al., 1996; Rubin, 2008; Viikari et al., 2012; Wettstein et al., 2012). However, bioconversion of cellulosic biomass into fermentable sugars is still the major technological and economical bottleneck to industrial implementation (Jørgensen et al., 2007; Ren et al., 2009; Viikari et al., 2012; Yang and Wyman, 2008).

Among the various agricultural and industrial residues, sugarcane bagasse is one of the most abundant lignocellulosic materials (Cardona et al., 2010; Pandey et al., 2000), especially in tropical countries since where the first cultivation started around 6,000 years ago. Sugarcane bagasse is a byproduct generated after the extraction of sucrose from sugarcane (*Saccharum officinarum*) is available on the spot as a potential feedstock for cellulosic biorefineries (Cardona et al., 2010). The enormous utilization of sugarcane for sugar and ethanol production in tropical countries such as Brazil, China and India, generate over 500 million metric tons of bagasse every year (Pandey et al., 2000). However, nearly half of the bagasse is used, mainly by direct burning, to generate heat and power for the plant operation (Brienzo, 2009; Shi et al., 2012) meaning that there still will be significant potential for using this material as feedstock for bolt-on plant to current sugar-based ethanol facilities. The material consists of fiber bundles and structural elements like plant vessels, epithelial cells and parenchyma, and represent a great morphological heterogeneity (Pandey et al., 2000). Like most other agricultural residues, bagasse consists of approximately 75% polysaccharides which are mainly composed of cellulose and hemicellulose. A low ash content, however, of 1.9% offers numerous advantages compared with others agro-based residues to the bioprocessing industries (Cardona et al., 2010).

Bioprocessing of lignocellulosic materials requires a pretreatment step in order to overcome the biomass recalcitrance for subsequent processing. Pretreatment is the most economically expensive step that hinders the industrial application of lignocellulose-based biorefineries (Alvira et al., 2010; Björnsson et al., 2012; Mosier et al., 2005). Numerous pretreatment methods, including physical (Lin et al., 2010), chemical (Martin et al., 2007; Zhang et al., 2012a; Zhu et al., 2012), thermal (Kumar et al., 2011; Yan et al., 2009), thermochemical (Biswas et al., 2012; Converse et al., 1989; Otieno and Ahring, 2012; Rana et al., 2012), and biological (Singh et al., 2008; Zhang et al., 2012b), have been reported, reviewed and published (Alvira et al., 2010; Björnsson et al., 2012; Chiaramonti et al., 2012; Hendriks and Zeeman, 2009; Mosier et al., 2005; Taherzadeh and Karimi, 2008; Wyman et al., 2005). In general, the purpose of pretreatment is to disrupt the complex cell wall structure, to solubilize the hemicellulose and to

render the cellulose accessible to facilitate enzymatic hydrolysis into the monomeric sugars.

The hydrolysate obtained from the pretreatment and/or enzymatic hydrolysis can be used as feedstock for microbial conversion into fuel products ethanol and butanol besides chemicals. Depending on the pretreatment severity, undesirable compounds can be formed during this process that may inhibit the microbial growth, decreasing yield and productivity (Palmqvist and Hahn-Hägerdal, 2000). Therefore, optimization of parameters of the process is necessary for each type of feedstock to improve the process efficiency.

Wet explosion (WEx), a thermo-chemical pretreatment process combining steam explosion and wet oxidation has previously shown excellent results in destruction of the lignocellulosic structure to enable the hydrolysis for subsequent bioconversion process (Ahring and Munck, 2006; Biswas et al., 2012; Georgieva et al., 2008; Rana et al., 2012). The process involves treatment of biomass at a concentration of up to 35% at temperatures above 150 °C with addition of air, O<sub>2</sub> or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) as a catalyst. Besides the obvious parameters of the pretreatment that include temperature, pressure and residence time, addition of external oxygen/air may enhance the severity of the pretreatment. As a catalyst, oxygen/air are favorable over any other expensive, toxic, corrosive and hazardous chemicals that are under investigation to perform the pretreatment of biomass (Sendich et al., 2008; Taherzadeh and Karimi, 2008) because of its cleanness as an oxidant and also have the great advantage of not leaving any residues in the hydrolysate that require additional sophisticated steps to neutralize.

In the wet explosion pretreatment of biomass, addition of oxygen acts as a strong oxidant which mainly reacts on lignin which will end as phenolics in the aqueous phase. Major fractions of the hemicellulose will be solubilized, thus improving the efficiency of the enzymatic hydrolysis by increasing the surface area. Furthermore, acetic acid is formed by de-esterification of the acetyl groups in xylans and by oxidation resulting in an increased acetic acid concentration which dissolves more hemicellulose in the aqueous phase. These combined factors result in an overall reduction in pH. The oxidative reaction also affects the cellulose which further increases the accessibility of the enzyme. Thus, recovery of the polysaccharides can be improved significantly by optimizing the conditions of pretreatment on for instance sugarcane bagasse. Furthermore, optimization of the cellulase enzymes and the enzyme to cellulose loading can improve the enzymatic hydrolysis of lignocellulosic material (Sun and Cheng, 2002).

Our present work is aimed to evaluate the optimal conditions for pretreating sugarcane bagasse using the WEx pretreatment method. Compared to previous work by Martín et al.

(2007, 2008a) on wet oxidation of sugarcane bagasse using 12 bar of oxygen with alkaline addition, our present work show significant improvements.

## **2. Materials and methods**

### *2.1. Substrate*

Sugarcane bagasse was obtained from Lafourche Sugar LLC, Thibodaux, LA, kindly provided by Edward Richard, Sugarcane Research Unit, USDA-ARS. Upon receiving, the bagasse was dried for two weeks at ambient temperature to reduce the moisture content to below 10%. It was milled, screened (0.2 mm), homogenized in a single lot and stored at ambient temperature until needed.

### *2.2. Wet explosion pretreatment*

Wet explosion pretreatment was performed at WSU pretreatment pilot plant using a custom-built 10 L stainless steel batch reactor (Rana et al., 2012) for disrupting the lignocellulosic matrix and fractioning the lignin and hemicellulosic components. The system is equipped with a stirrer and a 100 L subsequent flash tank connected to the reactor. The wet exploded material is accumulated in the flash tank after the pretreatment. The reactor is heated with an external oil heater. Temperature, pressure and steering speed of the motor are regulated and recorded electronically.

For pretreatments carried out with O<sub>2</sub>, the reactor was first flashed twice with O<sub>2</sub> to ensure an O<sub>2</sub>-only environment by removing the air. The reactor was hermetically closed, 6 bar of O<sub>2</sub> was then purged into the reactor and the reactor was heated to the desired temperature. Biomass was treated for 10 minutes at the constant temperature and pressure (Table 1). The pretreatment is terminated by sudden explosive decompression. In each batch of the pretreatment experiments, 640 g of oven dry bagasse was mixed with 3343 g of tap water, to reach a solids concentration of 16.0% (w/w), prior to impregnation and performing the pretreatment. Solid filter cake and liquid fractions were separated from the slurry by vacuum filtration. The samples were sealed in plastic containers and a portion of each sample was used for analysis while the remaining portions were kept frozen until further use.

### *2.3. Analysis of solid fraction*

The filter cake samples derived from the separation of the wet exploded materials were dried in a climatization chamber (Binder BD 240, NY, USA) at 37 °C and 65% relative humidity for

Table 1: Experimental conditions used for wet explosion and steam explosion of sugarcane bagasse at 16% dry matter concentration for 10 minutes.

Run	Temperature, °C	O <sub>2</sub> pressure, bar	End pressure, bar
B-155	155	0	5
B-155-O <sub>2</sub>	155	6	10
B-170	170	0	7
B-170-O <sub>2</sub>	170	6	17
B-185	185	0	11
B-185-O <sub>2</sub>	185	6	19
B-200	200	0	14
B-200-O <sub>2</sub>	200	6	22

48 hours to achieve constant weight with a moisture content below 10% according to National Renewable Energy Laboratory (NREL) (Hames et al., 2008). Dried samples were first made soluble with 72% (w/w) sulfuric acid at 30 °C in water-bath for 1 hour followed by dilute acid (4%, w/w) hydrolysis at 121 °C by autoclavation, modified from Sluiter et al. (2008). The amount of glucan, xylan, galactan, arabian, mannan, acetyl, soluble lignin, insoluble lignin, and structural inorganics were analyzed by the two step hydrolysis. Soluble lignin was analyzed by spectrophotometer (Jenway 6405 UV/Visible, NJ, USA) using a wavelength of 240 nm within 6 hours of hydrolysis. In parallel, analysis of total solids (TS), volatile solids (VS) and ash content were determined as described by Eaton and Franson (2005).

#### 2.4. Analysis of liquid fraction

The contents of free sugars, acids and degradation products in hemicellulose liquid fractions were determined using HPX-87H column in HPLC (Section 2.6). Total sugar monomers in the hemicellulose liquid fraction were determined by dilute acid hydrolysis of the oligomers in the hydrolysate. The hydrolysis was carried out in 4% H<sub>2</sub>SO<sub>4</sub> (w/w) at 121 °C for 10 minutes (Bjerre et al., 1996). This method allows the precipitation of the sulphate ions by an equivalent amount of Ba(OH)<sub>2</sub> which is then removed by centrifugation. The pH was determined using InLab<sup>®</sup> Micro combination pH electrode with precise measurement values ( $\pm 0.001$  pH).

#### 2.5. Enzymatic hydrolysis of washed solids

An equivalent amount of 30 g dry solid of each cake sample was mixed with 500 mL of DI water, vigorously mixed and soaked overnight at ambient temperature (22 °C) prior to vacuum filtration (0.45  $\mu$ m millipore). Approximately 160 mg of dry solid of washed samples were suspended in 200  $\mu$ L of 1M citrate buffer (pH 4.8), 20  $\mu$ L of sodium azide with the calculated

amount of enzymes mixture yielding 8% dry solid content. A mixture of two commercial enzymes Cellic<sup>®</sup>CTec2 and Cellic<sup>®</sup>HTec2 (Novozymes, USA) were used in a ratio of 85:15 (% v/v), respectively, to determine the enzymatic convertibility into sugar monomers. This enzymes ratio was found to be optimal for assessing enzymatic convertibility of the wet exploded bagasse (Biswas et al., [Unpublished]). Enzyme loadings at 12.4 and 22.0 mg enzyme protein (EP)/g cellulose were evaluated with a 15% replacement of Cellic<sup>®</sup>CTec2 with Cellic<sup>®</sup>HTec2 for both enzyme loadings. Hydrolysis was carried out in 2.5 mL Eppendorf tube with an active volume of 2 mL, DI water was added to adjust the dry solid %. The reaction mixtures were incubated in thermomixture (Thermomixer<sup>®</sup> R, Eppendorf North America, Hauppauge, NY) at 50 °C shaking at 1400 rpm for 96 hours. The hydrolysis experiments were performed in triplicates, while the controls for each sample, with DI water instead of enzymes to determine free sugar release, were performed in duplicates. The mean value obtained from the controls was subtracted from the respective values of the sample with enzymes. At the termination of the hydrolysis, the hydrolysate was centrifuged at 14000 rpm for 30 mins at 4 °C and filtered (0.45  $\mu$ m). The sugar monomers were quantified by HPLC using HPX-87P column (Section 2.6). The protein content in each enzyme was analyzed with BCA Protein Assay Kit using the company's procedure (Thermo Fisher Scientific, Rockford, IL, USA).

## *2.6. Analytical procedures*

Samples were always filtered (0.45  $\mu$ m PTFE membrane, Acrodisc<sup>®</sup> Syringe Filters, 13mm, Pall<sup>®</sup> Life Sciences, USA) prior to HPLC analysis. Samples were quantified using HPLC equipped with refractive index and UV visible detector. Glucose, xylose, arabinose, acetic acid, HMF and furfural were analyzed on an Aminex HPX-87H column (Bio-Rad, Hercules, USA) at 60 °C with 4 mM H<sub>2</sub>SO<sub>4</sub> as an eluent with a flow rate of 0.6 mL/minute. Monomeric sugars glucose, xylose, galactose, arabinose, mannose in acid and enzymatic hydrolysates were analyzed using an Aminex HPX-87P column (Bio-Rad, Hercules, USA) at 83 °C with a flow rate of 6.0 mL/minute using deionized water (Barnstead Nanopure, IA, USA) as mobile phase.

## *2.7. Calculation and statistical method*

The glucose yield (%), xylose yield (%) and enzymatic efficiency, EE (%) were calculated using the following equations;



$$\text{Glucose yield (\%)} = \frac{[\text{Glucose}], \text{ g/L}}{(\text{Solids} \times \text{Glucan\%} \times 1.11), \text{ g/L}} \times 100\% \quad (1)$$

$$\text{Xylose yield (\%)} = \frac{[\text{Xylose}], \text{ g/L}}{(\text{Solids} \times \text{Xylan\%} \times 1.14), \text{ g/L}} \times 100\% \quad (2)$$

$$\text{EE (\%)} = \frac{[\text{Glucose}], \text{ g/L} + [\text{Xylose}], \text{ g/L}}{(\text{Solids} \times \text{Glucan\%} \times 1.11) + (\text{Solids} \times \text{Xylan\%} \times 1.14), \text{ g/L}} \times 100\% \quad (3)$$

In which [Glucose] and [Xylose] represent the concentrations of respective sugars after the enzymatic hydrolysis where the mean value of respective controls was deducted for each sample; 1.11 and 1.14 are the coefficient of glucose and xylose obtained from glucan and xylan, respectively. Only glucose and xylose were considered in determination of enzymatic efficiency because they are the predominant sugars present in bagasse hydrolysate.

### 3. Results and discussion

In this study, we investigated the pilot scale performance of WEx pretreatment applying different conditions (Table 1) on sugarcane bagasse in terms of sugar yields, formation of degradation products and efficiency in enzymatic hydrolysis. Additionally, two different loadings of enzyme mixtures were evaluated to maximize the enzymatic efficiency (Eq. 3). In particular, a suitable set of WEx pretreatment parameters to improve overall recovery and sugar yields, while minimizing the formation of degradation products as well as lowering the costs of enzymes for a biorefinery has been focused.

#### 3.1. Characterization of raw sugarcane bagasse

The composition of the raw milled bagasse is depicted in table 2. The composition of sugarcane bagasse may vary depending on the growing season, harvesting procedure, processing method, growing location as well as analytical procedures. The carbohydrate content accounts for 58.2% of the dry bagasse. Glucan, which is mainly cellulose was the major component making up 33.8% followed by xylan at 22.0%, the major hemicellulose constituent. Glucan content was slightly lower than the reported literature value of 37.4% cellulose while the hemi-

cellulose and lignin content were similar to previously reported values of 23.7% and 25.1%, respectively (Fuentes et al., 2011).

Table 2: Main components of sugarcane bagasse used in this study.

Components	% dry weight <sup>a</sup>
Holocellulose	58.2
Glucan	33.8
Xylan	22.0
Arabinan	2.4
Total lignin	23.9
Acid-soluble lignin	1.4
Acid-insoluble lignin	22.5
Acetyl	2.6

<sup>a</sup>Dry mater of the bagasse was 97.4% (volatile solids 93.7% and ash 6.3%)

### 3.2. Effect of wet explosion conditions on the composition of the wet exploded material

The conditions used for WEx pretreatment (Table 1) were chosen based on the conditions that were favorable for WEx pretreatment of digested fibers (Biswas et al., 2012) and literature (Klinke et al., 2003; Martín et al., 2008b; Palmqvist and Hahn-Hägerdal, 2000). The cracking with vapor or O<sub>2</sub> together with explosive decompression typically cause double effects in the wet explosion pretreatment. Thickness of the wet exploded bagasse slurry obtained in pretreatment B-170-O<sub>2</sub> (170 °C with O<sub>2</sub>) and in pretreatment B-200 (200 °C without O<sub>2</sub>) were analogous, indicating that O<sub>2</sub> has significant influence in solubilizing the lignocellulose constituents during the WEx pretreatment. The slurry obtained in pretreatment B-200-O<sub>2</sub>, where solubilization is more pronounced, resulting in less viscous material (Figure 1). The orders of more solubilized materials were untreated bagasse <<< B-155 < B-170 < B-155-O<sub>2</sub> ≤ B-185 < B-200 ≤ B-170-O<sub>2</sub> < B-185-O<sub>2</sub> < B-200-O<sub>2</sub>. The orders also reflect the color of the wet exploded materials from light brown to dark brown indicating the severity of the pretreatment under different conditions.

The pH of untreated bagasse was 5.85 prior to the WEx pretreatment. A decrease of pH to 2.93–4.35 was observed for all of the pretreatment conditions tested. Lowest final pH (2.93) was observed in the pretreatment B-200-O<sub>2</sub>, which was the most severe condition tested (Table 3). The concentration of acetic acid is proportionally related to the pH and severity of the pretreatment, with more sever pretreatment conditions resulting in higher concentration of acids, thus lowering the pH.

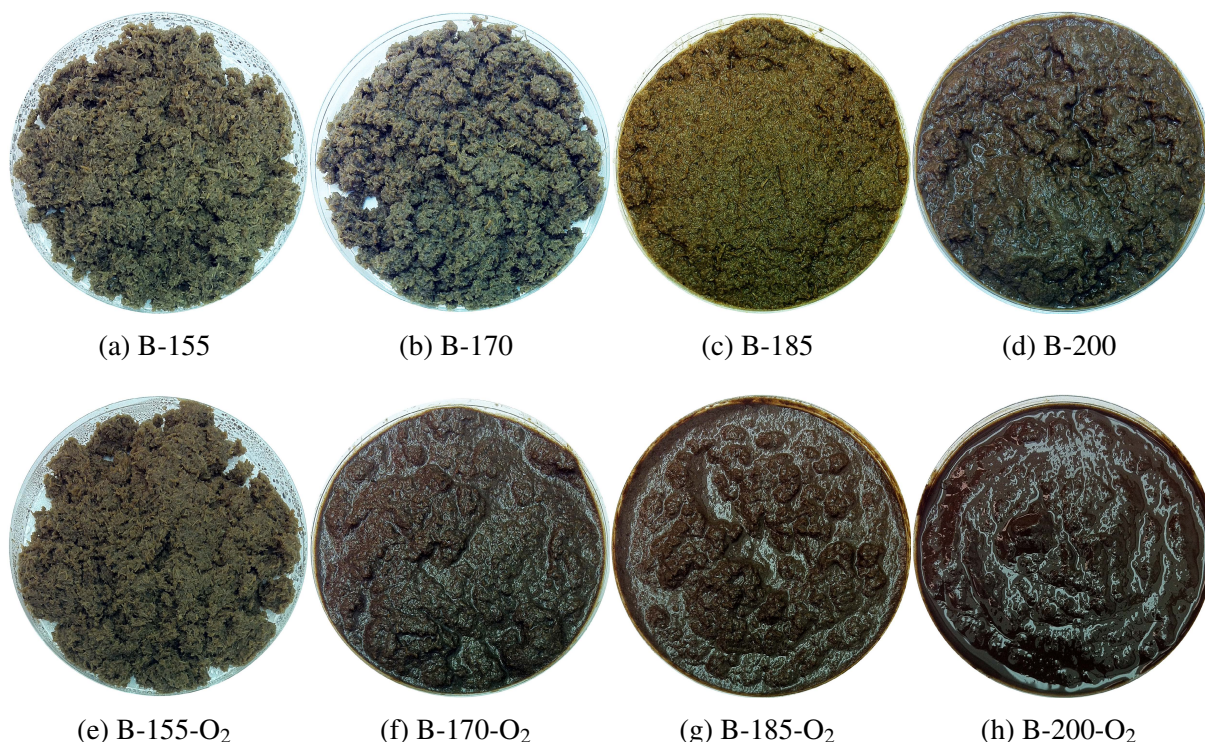


Figure 1: Wet exploded bagasse in different pretreatment conditions (a - h).

The higher dry matter content in the solid cakes (Table 3) indicates that close to half of the solids were solubilized in most severe pretreatment conditions. The compositional analysis of the solid cake samples revealed that the percentage of glucan has been increased in all solid cake samples (Figure 2a), ranging from 40.2–59.2 (% dry weight basis). This highest glucan content in solid cake was found in pretreatment B-185-O<sub>2</sub>. This value is comparable to the wet oxidation of sugarcane bagasse using 1 L reactor with 5.63% dry matter at 195 °C for 15 minutes, with 2 g of Na<sub>2</sub>CO<sub>3</sub> and 12 bar O<sub>2</sub> (Martín et al., 2008a).

After dilute acid hydrolysis of the liquid fractions, xylose was found to be the predominant sugar in pretreatment B-170-O<sub>2</sub>, B-185, B-185-O<sub>2</sub> and B-200 resulting 15.2, 19.9, 26.0 and 26.6 g/L, respectively. Hence, pretreatment B-185-O<sub>2</sub> is more favorable as the highest amount (12.1 g/L) of xylose was detected as free sugar (Figure 2b). Furthermore, during pretreatment, glucose can also be hydrolyzed both from the cellulose and from the side-chains of hemicellulose. Considerably higher concentration of glucose (5.1 and 5.8 g/L) were found in the liquid fractions of pretreatment B-185-O<sub>2</sub> and B-200-O<sub>2</sub>, respectively (Table 3). We observed that the glucose solubilization was improved at a temperature above 185 °C only when the oxygen was added. The differences in the sugar concentrations of the liquid fraction before and after dilute acid hydrolysis indicating incomplete hydrolysis of the polysaccharides into monomeric sug-

ars. Hemicellulose has been almost completely solubilized under condition B-200-O<sub>2</sub>, leaving only 2.4% xylan in the solid cake (dry basis). However, xylose concentration was found to be lower after both free and total sugar analysis of the liquid fraction under this condition (B-200-O<sub>2</sub>) (Table 3). This can be explained by the fact that the xylose has been further degraded into furfural, resulting in higher concentration (3.3 g/L) in the liquid fraction (Figure 2c).

Additionally, the concentrations of acetate (7.6 g/L) and HMF (1.0 g/L) were higher in pretreatment B-200-O<sub>2</sub>, indicating the formation of the weak acid due to de-acetylation of hemicellulose. The formation of degradation products is unavoidable under the harshest condition. The formation of HMF and furfural during pretreatment, due to the dehydration of glucose and xylose respectively (Binder, 2010; Palmqvist and Hahn-Hägerdal, 2000), was also observed in pretreatment B-185-O<sub>2</sub> and B-200, but comparatively in lower degrees (Figure 2c). Carboxylic acid formation from sugars, phenols and other compounds increased with increasing the severity of the pretreatment; this is well studied and reported elsewhere (Klinke et al., 2003; Martín et al., 2008b; Palmqvist and Hahn-Hägerdal, 2000). Although the degradation products including carboxylic acids, HMF and furfural are undesirable for the following fermentation, these inhibitors can be metabolized and tolerated by *S. cerevisiae* and/or *P. stipitis* to some extent

under both aerobic and anaerobic conditions (Palmqvist and Hahn-Hägerdal, 2000; Wan et al.,

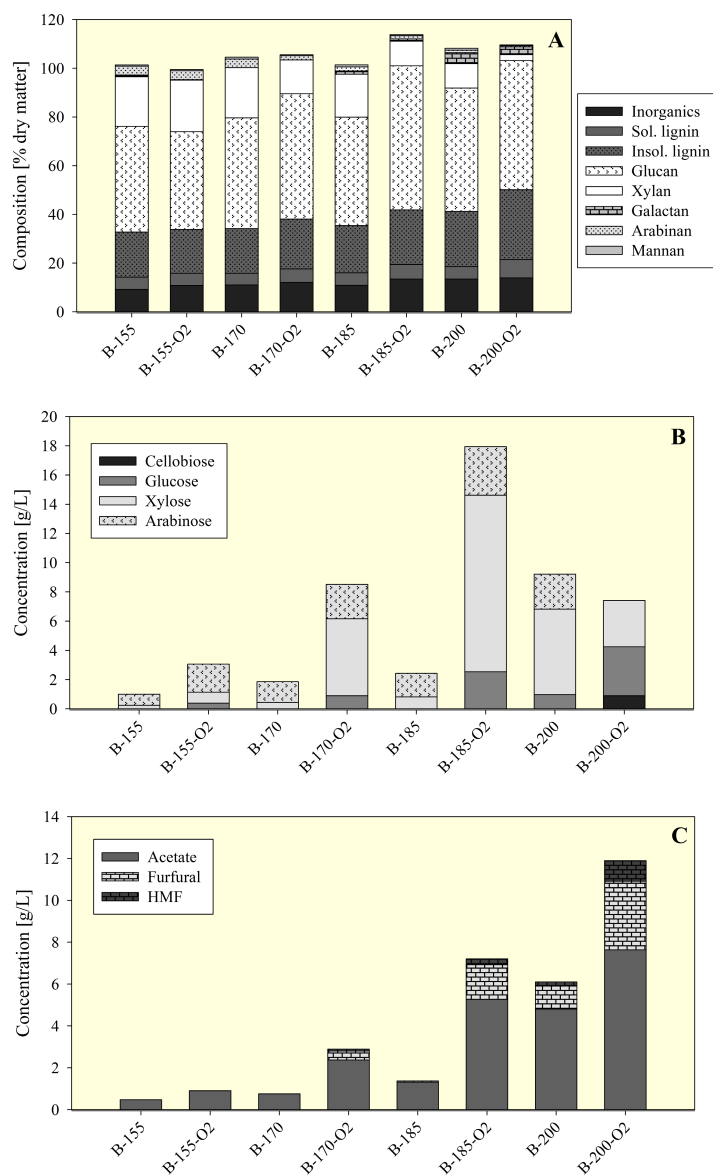


Figure 2: Analysis of solid and liquid fractions of the wet exploded bagasse under different pretreatment conditions: A. composition of solid cakes; B. free sugars in the liquid fractions; and C. concentration of degradation compounds.

2012). The combined inhibitory effects of the degradation products are likely to be greater than seen for the single compound. Pretreatment B-200-O<sub>2</sub> exerted a clear effect on the formation of inhibitory products where detoxification can be necessary for efficient enzymatic hydrolysis and/or subsequent biological conversion processes.

### *3.3. Effect of enzyme loadings and wet explosion conditions on the enzymatic hydrolysis*

Enzymatic efficiency of washed solid cakes was assessed under two different enzyme loadings (12.4 and 22.0 mg EP/g cellulose) and compared with the untreated washed bagasse (Table 4). The overall glucose yield, xylose yield and enzymatic efficiency were calculated based on the experimental results following the equation 1, 2 and 3, respectively. In the previous study, kinetic behavior for the optimal two enzymes mixtures, dosage and optimal enzyme to dry matter ratio were evaluated on the whole slurry of wet exploded bagasse (Biswas et al., [Unpublished]). The enzymatic efficiency indicates the fraction of holocellulose (in this case only glucose and xylose) that was hydrolyzed into sugar monomers. The glucose and xylose yield values incorporate both the different wet explosion pretreatment condition and enzyme loadings in the subsequent hydrolysis. The enzyme protein (EP) content of the enzymes Cellic®CTec2 and Cellic®HTec2 determined prior to enzymatic hydrolysis were  $279 \pm 8$  and  $251 \pm 12$  mg EP/mL, respectively.

Wet explosion pretreatment was effective in all conditions tested. Enzymatic efficiency was found to be far greater than that of the untreated material at both enzyme loadings evaluated. However, overall sugar yields with the enzyme loading 12.4 mg EP/g cellulose was found to be significantly lower in all experiments showing that an enzyme loading of 22.0 mg EP/g cellulose is more optimal for the hydrolysis of the washed solid materials giving an overall maximum sugar yield of glucose ( $573.9 \pm 4.5$  g/Kg TS) for the pretreatment condition at 185 °C with oxygen (B-185-O<sub>2</sub>), followed by  $567.7 \pm 3.1$  g/Kg TS in B-200-O<sub>2</sub>. Therefore, the glucose yields under the pretreatments conducted with oxygen at a temperature of 185 °C and 200 °C are analogous. However, the cellulose hydrolysis were  $87.4 \pm 0.7\%$  and  $96.4 \pm 0.5\%$  of the theoretical maximum in B-185-O<sub>2</sub> and B-200-O<sub>2</sub>, respectively. Under both pretreatment conditions, glucose yields were greater than the yields obtained by de Moraes Rocha et al. (2011) in dilute mixed-acid pretreatment of sugarcane bagasse and also greater than most of the yields reviewed by Cardona et al. (2010). Similar results were reported by Martín et al. (2008b) with a 93.6% glucose recovery in the pretreatment of clover-ryegrass at 195 °C with 12 bar O<sub>2</sub> pressure for

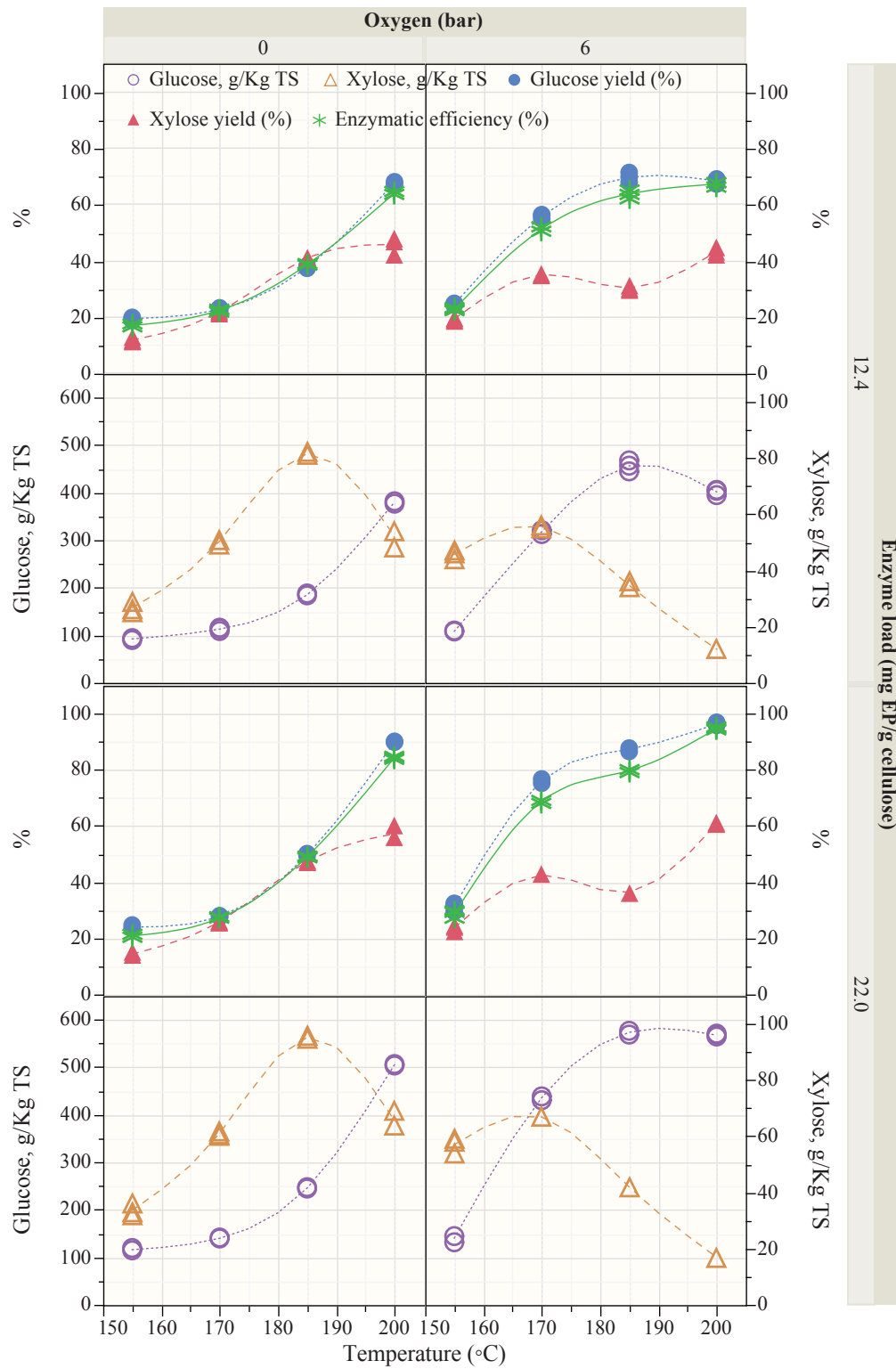


Figure 3: The effects of enzyme loading, pretreatment temperature and addition of oxygen on glucose and xylose yields, and enzymatic efficiency of washed wet exploded bagasse. The enzymatic hydrolysis conditions were enzyme loading of 12.4 and 22.0 mg EP/g cellulose at 50°C, pH of 4.8 for 96 hours.

10 minutes using a higher enzyme loading and a lower dry matter concentrations than us. It is noteworthy that our results show significant improvement in enzymatic hydrolysis of sugar-cane bagasse using wet explosion as a pretreatment method. Furthermore, the enzymes dosage (22.0 mg EP/g cellulose) used in this study represents a lower enzyme dosage than what has previously been reported (Dyk and Pletschke, 2012; Martín et al., 2008b; Monavari, 2011; Zhu et al., 2012). Pretreatments B-155, B-155-O<sub>2</sub>, B-170 and B-185 were not severe enough to improve the enzymatic hydrolysis significantly.

Although highest enzymatic efficiency  $94.8 \pm 0.5\%$  was achieved in pretreatment B-200-O<sub>2</sub>, the maximum release of total sugars (glucose + xylose)  $49.5 \pm 0.5$  g/L was obtained in pretreatment B-185-O<sub>2</sub> (Table 4). However, pretreatment B-200-O<sub>2</sub> may not be preferable due to the formation of acetate, HMF and furfural in higher concentration (Figure 2c). Inhibitory products formed during pretreatment under condition B-200-O<sub>2</sub> would be required for a successful bioconversion process, any need for detoxification could make the process economically unfeasible.

Enzymatic efficiency involves hydrolysis of both C5 and C6 sugar during processing of log-nocellulose hydrolysate. The glucose released ( $46.1 \pm 0.5$  g/L) in the hydrolysate of B-185-O<sub>2</sub> was among the highest. However, slightly lower xylose recovery from the washed solid of B-185-O<sub>2</sub> resulted in comparatively lower enzymatic efficiency,  $79.7 \pm 0.6\%$  (Figure 3). However, the glucose yield in the enzymatic hydrolysis of washed solid cake under pretreatment B-185-O<sub>2</sub> is comparable to that of B-200-O<sub>2</sub>. The highest amount of xylose released in the sample of pretreatment B-185 in both enzyme loadings (12.4 and 22.0 mg EP/g cellulose) due to high residual xylan (Figure 2a) in the washed dry solid (17.6%) after the pretreatment. In the contrary, enzymatic efficiencies in both the loadings were significantly lower. For the comparison purpose, the glucose yield, xylose yield and enzymatic efficiency of the untreated samples at an enzyme loading of 22.0 mg EP/g cellulose were  $16.5 \pm 0.6\%$ ,  $5.2 \pm 0.3\%$  and  $12.0 \pm 5.0\%$ , respectively.

Table 3: Characterization of the wet exploded material obtained in the pretreatments under different conditions.

	Bagasse <sup>d</sup>	B-155	B-155-O <sub>2</sub>	B-170	B-170-O <sub>2</sub>	B-185	B-185-O <sub>2</sub>	B-200	B-200-O <sub>2</sub>
<b>Slurry</b>									
Total solid, %	16.0	17.0	16.2	15.1	15.5	16.5	16.2	16.9	14.0
Volatile solid, %	15.0	15.2	14.3	13.2	13.7	14.6	14.0	14.7	12.0
Ash, %	1.0	1.8	1.8	1.9	1.9	1.9	2.2	2.1	1.9
<b>Solid fraction, filter cake</b>									
Dry matter, g 100 g <sup>-1</sup>	NA <sup>b</sup>	26.6 (100%)	29.8 (100%)	24.8 (100%)	27.7 (100%)	25.2 (100%)	28.4 (100%)	26.5 (100%)	21.3 (100%)
Glucan <sup>c</sup> , g 100 g <sup>-1</sup>	NA	11.5 (43.4%)	12.0 (40.2%)	11.3 (45.5%)	14.3 (51.5%)	11.2 (44.6%)	16.8 (59.2%)	13.4 (50.7%)	11.3 (53.1%)
Xylan, g 100 g <sup>-1</sup>	NA	5.4 (20.4%)	6.3 (21.1%)	5.1 (20.6%)	3.8 (13.9%)	4.4 (17.6%)	2.9 (10.1%)	2.7 (10.0%)	0.5 (2.4%)
Galactan, g 100 g <sup>-1</sup>	NA	0.2 (0.7%)	0.1 (0.2%)	0.0 (0.0%)	0.0 (0.0%)	0.3 (1.3%)	0.3 (0.9%)	1.2 (4.6%)	0.7 (3.2%)
Arabinan, g 100 g <sup>-1</sup>	NA	1.0 (3.7%)	1.1 (3.6%)	0.9 (3.5%)	0.5 (1.7%)	0.4 (1.7%)	0.4 (1.3%)	0.3 (1.0%)	0.1 (0.5%)
Mannan, g 100 g <sup>-1</sup>	NA	0.1 (0.5%)	0.2 (0.5%)	0.2 (0.8%)	0.1 (0.4%)	0.2 (0.8%)	0.1 (0.4%)	0.2 (0.8%)	0.1 (0.3%)
Soluble lignin, g 100 g <sup>-1</sup>	NA	1.3 (5.0%)	1.5 (5.1%)	1.2 (4.8%)	1.5 (5.5%)	1.3 (5.2%)	1.7 (6.0%)	1.4 (5.2%)	1.6 (7.4%)
Total lignin, g 100 g <sup>-1</sup>	NA	6.2 (23.5%)	6.9 (23.0%)	5.7 (23.1%)	7.2 (26.0%)	6.2 (24.5%)	8.1 (28.4%)	7.4 (27.8%)	7.7 (36.1%)
Ash, g 100 g <sup>-1</sup>	NA	2.4 (9.2%)	3.2 (10.8%)	2.7 (11.0%)	3.3 (12.1%)	2.7 (10.8%)	3.8 (13.5%)	3.6 (13.4%)	3.0 (14.0%)
<b>Total sugar, g 100 g<sup>-1</sup></b>	NA	18.2 (68.7%)	19.6 (65.6%)	17.5 (70.4%)	18.7 (67.5%)	16.6 (66.0%)	20.4 (72.0%)	17.8 (67.0%)	12.6 (59.5%)
<b>Total mass, g 100 g<sup>-1</sup></b>	NA	26.9 (101.4%)	29.7 (99.5%)	25.9 (104.5%)	29.2 (105.6%)	25.6 (101.4%)	32.3 (113.8%)	28.7 (108.3%)	23.3 (109.6%)
<b>Liquid fraction, filtrate</b>									
pH	5.85	4.35	3.45	4.02	3.12	3.81	3.05	3.40	2.93
<b>Total sugars<sup>d</sup></b>									
Glucose, g L <sup>-1</sup>	NA	ND <sup>e</sup>	2.0	3.1	2.7	2.5	5.1	2.3	5.8
Xylose, g L <sup>-1</sup>	NA	ND	6.2	4.9	15.2	19.9	26.0	26.6	4.9
Arabinose, g L <sup>-1</sup>	NA	ND	1.9	1.9	1.8	3.1	2.9	2.3	4.0
<b>Free sugars</b>									
Cellobiose, g L <sup>-1</sup>	NA	BDL <sup>f</sup>	BDL	BDL	BDL	BDL	BDL	BDL	0.9
Glucose, g L <sup>-1</sup>	NA	BDL	0.4	BDL	0.9	BDL	2.5	1.0	3.4
Xylose, g L <sup>-1</sup>	NA	0.2	0.7	0.4	5.3	0.8	12.1	5.8	3.2
Arabinose, g L <sup>-1</sup>	NA	0.8	1.9	1.4	2.4	1.6	3.3	2.4	BDL
Total sugar, g L <sup>-1</sup>	NA	1.0	3.1	1.9	8.5	2.4	17.9	9.2	7.4
Acetate, g L <sup>-1</sup>	NA	0.5	0.9	0.8	2.4	1.3	5.3	4.8	7.6
Furfural, g L <sup>-1</sup>	NA	BDL	BDL	BDL	0.4	0.1	1.7	1.1	3.3
HMF, g L <sup>-1</sup>	NA	BDL	BDL	BDL	0.1	BDL	0.3	0.2	1.0

<sup>d</sup>Bagasse was mixed with tap water to achieve a dry solid content of 16.0% to impregnate WEx reactor.

<sup>b</sup>NA = not applicable.

<sup>c</sup>Percentage of dry solid cake material shown in parentheses.

<sup>d</sup>after dilute acid hydrolysis

<sup>e</sup>ND = not determined.

<sup>f</sup>BDL = below the detection limit.



Table 4: Effect of the pretreatment conditions and enzyme loading on enzymatic hydrolysis of the washed solid fractions of wet exploded sugarcane bagasse.

Enzyme loading and hydrolysis (96 h)		Untreated	B-155	B-155-O <sub>2</sub>	B-170	B-170-O <sub>2</sub>	B-185	B-185-O <sub>2</sub>	B-200	B-200-O <sub>2</sub>
<b>12.4 mg EP/g cellulose</b>										
Glucose, g Kg <sup>-1</sup> TS		49.9±1.7	92.4±2.5	108.3±1.3	113.1±3.5	317.9±4.5	184.9±2.4	456.6±11.2	380.0±2.8	401.6±5.8
Xylose, g Kg <sup>-1</sup> TS		9.8±0.9	26.85±1.9	45.9±1.5	50.3±1.0	55.3±0.5	81.9±0.8	34.6±0.9	52.0±3.4	11.9±0.4
Glucose, g L <sup>-1</sup>		4.0±0.1	7.5±0.2	8.7±0.1	9.1±0.3	25.5±0.3	14.9±0.2	36.7±0.6	30.7±0.3	32.6±0.2
Xylose, g L <sup>-1</sup>		0.8±0.1	2.2±0.2	3.7±0.1	4.1±0.1	4.4±0.0	6.6±0.0	2.8±0.1	4.2±0.3	1.0±0.0
Released glucose + xylose, g L <sup>-1</sup>		4.8±0.2	9.6±0.3	12.4±0.2	13.2±0.4	29.9±0.4	21.6±0.3	39.5±0.7	34.9±0.3	33.6±0.3
Glucose yield, %		13.3±0.5	19.2±0.5	24.3±0.3	22.4±0.7	55.6±0.8	37.4±0.5	69.5±1.7	67.6±0.5	68.2±1.0
Xylose yield, %		3.9±0.4	11.5±0.8	19.0±0.6	21.4±0.4	35.0±0.3	40.9±0.4	30.0±0.8	45.5±3.0	43.0±1.4
Enzymatic efficiency, %		9.5±0.4	16.7±0.6	22.4±0.4	22.1±0.6	51.1±0.7	38.4±0.4	63.6±1.6	63.8±0.5	67.1±1.0
<b>22.0 mg EP/g cellulose</b>										
Glucose, g Kg <sup>-1</sup> TS		61.9±4.6	116.1±3.1	140.2±7.0	140.2±1.5	436.3±4.8	245.8±2.3	573.9±4.5	505.9±1.5	567.7±3.1
Xylose, g Kg <sup>-1</sup> TS		13.1±1.5	33.6±1.8	57.2±2.7	60.7±0.8	67.4±0.1	95.2±0.4	42.0±0.1	65.4±3.0	16.8±0.1
Glucose, g L <sup>-1</sup>		5.0±0.4	9.4±0.2	11.2±0.6	11.3±0.1	35.2±0.3	19.8±0.2	46.1±0.5	40.9±0.2	45.7±0.2
Xylose, g L <sup>-1</sup>		1.0±0.1	2.7±0.1	4.6±0.2	4.9±0.1	5.4±0.0	7.7±0.0	3.4±0.0	5.3±0.3	1.4±0.0
Released glucose + xylose, g L <sup>-1</sup>		6.0±0.5	12.1±0.3	15.8±0.8	16.1±0.2	40.6±0.3	27.5±0.2	49.5±0.5	46.2±0.5	47.0±0.3
Glucose yield, %		16.5±0.6	24.1±0.7	31.4±1.6	27.8±0.3	76.3±0.8	49.7±0.5	87.4±0.7	90.0±0.3	96.4±0.5
Xylose yield, %		5.2±0.3	14.4±0.8	23.7±1.1	25.8±0.4	42.7±0.0	47.5±0.2	36.4±0.1	57.2±2.7	60.9±0.4
Enzymatic efficiency, %		12.0±0.5	20.9±0.7	28.7±1.4	27.2±0.3	69.0±0.7	49.0±0.3	79.7±0.6	84.4±0.5	94.8±0.5

#### 4. Conclusion

This study investigated a suitable process conditions for wet explosion pretreatment of sugarcane bagasse in a biorefinery concept. Wet explosion is a promising pretreatment method for improving the enzymatic hydrolysis of lignocellulose into monomeric sugars for microbial processing into ethanol, chemicals and value added products. Temperature and addition of oxygen were both proven to be important factors in optimizing the pretreatment conditions. Enzymatic hydrolysis was optimized with washed solid fractions of wet exploded bagasse and the hydrolysis was significantly improved at temperatures over 170 °C and oxygen further had an effect on hydrolysis.

Results of this study revealed that the suitable condition for wet explosion pretreatment of sugarcane bagasse is 185 °C with 6 bar O<sub>2</sub> pressure for 10 minutes. The solubilization of hemicellulose and lignin enriched the glucan content (59.2%, dry weight) in wet exploded solid cake under this pretreatment condition. The enzymatic hydrolysis of the washed solid cake yielded glucose up to 573.9±4.5 g/Kg TS and 87.4±0.7% of the theoretical maximum with the enzyme loading of 22.0 mg EP/g cellulose. Besides, highest total sugar (glucose, xylose and arabinose, 34.0 g/L) was found in liquid fraction in pretreatment at 185 °C with 6 bar O<sub>2</sub> pressure. Although, comparable performance was observed in enzymatic hydrolysis of washed cake (glucose yield of 96.4±0.5%) under the condition at 200 °C with 6 bar O<sub>2</sub> pressure for 10 minutes, formation of comparably higher concentration of degradation products and possible need for detoxification of the hydrolysate could be economically unfeasible.

#### Acknowledgement

The authors would like to express gratitude to the Strategic Research Council for support to the Biorefinery project which has funded this work. We further thank to Dr. Keith Thomsen at BSEL and Dr. Edward Richard at the USDA for the assistance in collection of the sugarcane bagasse used in this study.

#### 5. References

- Ahring, B., Jensen, K., Nielsen, P., Bjerre, A., Schmidt, A., 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. *Bioresource Technology* 58 (2), 107–113.
- Ahring, B., Munck, J., Mar. 30 2006. Method for treating biomass and organic waste with the purpose of generating desired biologically based products. WO 2006/032282 A1.
- Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology* 101 (13), 4851–4861.

- Binder, J.B. & Raines, R., 2010. Fermentable sugars by chemical hydrolysis of biomass. *Proceedings of the National Academy of Sciences* 107 (10), 4516–4521.
- Biswas, R., Ahring, B., Uellendahl, H., 2012. Improving biogas yields using an innovative concept for conversion of the fiber fraction of manure. *Water Science & Technology* 66 (8), 1751–1758.
- Biswas, R., Marczak, M., Uellendahl, H., Ahring, B., [Unpublished]. Search for optimal combinations and ratios of enzymes for the hydrolysis of wet exploded bagasse. In preparation.
- Bjerre, A., Olesen, A., Fernqvist, T., Plöger, A., Schmidt, A., 1996. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. *Biotechnology and Bioengineering* 49 (5), 568–577.
- Björnsson, L., Svensson, S., Monavari, S., Kreuger, E., Zacchi, G., Jun. 21 2012. Pretreatment of non-wood lignocellulosic material. US Patent 20,120,156,727.
- Brienzo, M.; Siqueira, A. . M. A., 2009. Search for optimum conditions of sugarcane bagasse hemicellulose extraction. *Biochemical Engineering Journal* 46 (2), 199–204.
- Cardona, C., Quintero, J., Paz, I., 2010. Production of bioethanol from sugarcane bagasse: status and perspectives. *Bioresource Technology* 101 (13), 4754–4766.
- Chiaromonti, D., Prussi, M., Ferrero, S., Oriani, L., Ottonello, P., Torre, P., Cherchi, F., 2012. Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method. *Biomass and Bioenergy* In press (0), 0–0.
- Converse, A., Kwarteng, I., Grethlein, H., Ooshima, H., 1989. Kinetics of thermochemical pretreatment of lignocellulosic materials. *Applied Biochemistry and Biotechnology* 20 (1), 63–78.
- de Moraes Rocha, G. J., Martin, C., Soares, I. B., Maior, A. M. S., Baudel, H. M., de Abreu, C. A. M., 2011. Dilute mixed-acid pretreatment of sugarcane bagasse for ethanol production. *Biomass and Bioenergy* 35 (1), 663–670.
- Dyk, J. V., Pletschke, B., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy. *Biotechnology Advances* (0), –.
- Eaton, A., Franson, M., 2005. Standard methods for the examination of water & wastewater. Amer Public Health Assn.
- Fuentes, L., Rabelo, S., Filho, R., Costa, A., 2011. Kinetics of lime pretreatment of sugarcane bagasse to enhance enzymatic hydrolysis. *Applied Biochemistry and Biotechnology* 163 (5), 612–625.
- Georgieva, T., Hou, X., Hilstrøm, T., Ahring, B., 2008. Enzymatic hydrolysis and ethanol fermentation of high dry matter wet-exploded wheat straw at low enzyme loading. *Biotechnology for Fuels and Chemicals*, 553–562.
- Hames, B., Ruiz, R., Scarlata, C., Sluiter, A., Sluiter, J., Templeton, D., 2008. Preparation of samples for compositional analysis. *Laboratory Analytical Procedure (LAP)*.
- Hendriks, A., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology* 100 (1), 10–18.
- Jørgensen, H., Kristensen, J., Felby, C., 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioproducts and Biorefining* 1 (2), 119–134.

- Klinke, H., Olsson, L., Thomsen, A., Ahring, B., 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: Wet oxidation and fermentation by yeast. *Biotechnology and Bioengineering* 81 (6), 738–747.
- Kumar, S., Kothari, U., Kong, L., Lee, Y., Gupta, R., 2011. Hydrothermal pretreatment of switchgrass and corn stover for production of ethanol and carbon microspheres. *Biomass and Bioenergy* 35 (2), 956–968.
- Lin, Z., Huang, H., Zhang, H., Zhang, L., Yan, L., Chen, J., 2010. Ball milling pretreatment of corn stover for enhancing the efficiency of enzymatic hydrolysis. *Applied Biochemistry and Biotechnology* 162 (7), 1872–1880.
- Martin, C., Alriksson, B., Sjöde, A., Nilvebrant, N., Jönsson, L., 2007. Dilute sulfuric acid pretreatment of agricultural and agro-industrial residues for ethanol production. *Applied Biochemistry and Biotechnology* 137 (1), 339–352.
- Martín, C., Klinke, H., Thomsen, A., 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. *Enzyme and Microbial Technology* 40 (3), 426–432.
- Martín, C., Marcet, M., Thomsen, A., et al., 2008a. Comparison between wet oxidation and steam explosion as pretreatment methods for enzymatic hydrolysis of sugarcane bagasse. *BioResources* 3 (3), 670–683.
- Martín, C., Thomsen, M., Hauggaard-Nielsen, H., BelindaThomsen, A., 2008b. Wet oxidation pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation of clover-ryegrass mixtures. *Bioresource Technology* 99 (18), 8777–8782.
- Monavari, S.; Galbe, M. . Z. G., 2011. Influence of impregnation with lactic acid on sugar yields from steam pretreatment of sugarcane bagasse and spruce, for bioethanol production. *Biomass and Bioenergy*.
- Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96 (6), 673–686.
- Otieno, D. O., Ahring, B. K., 2012. The potential for oligosaccharide production from the hemicellulose fraction of biomasses through pretreatment processes: xylooligosaccharides (XOS), arabinooligosaccharides (AOS), and mannoooligosaccharides (MOS). *Carbohydrate Research* 360 (0), 84 – 92.
- Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresource Technology* 74 (1), 17–24.
- Pandey, A., Soccol, C., Nigam, P., Soccol, V., 2000. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresource Technology* 74 (1), 69–80.
- Rana, D., Rana, V., Ahring, B. K., 2012. Producing high sugar concentrations from loblolly pine using wet explosion pretreatment. *Bioresource Technology* 121 (0), 61–67.
- Ren, N., Wang, A., Cao, G., Xu, J., Gao, L., 2009. Bioconversion of lignocellulosic biomass to hydrogen: potential and challenges. *Biotechnology Advances* 27 (6), 1051–1060.
- Rubin, E., 2008. Genomics of cellulosic biofuels. *Nature* 454 (7206), 841–845.
- Sendich, E., Laser, M., Kim, S., Alizadeh, H., Laureano-Perez, L., Dale, B., Lynd, L., 2008. Recent process improvements for the ammonia fiber expansion (AFEX) process and resulting reductions in minimum ethanol selling price. *Bioresource Technology* 99 (17), 8429–8435.
- Shi, J., Yang, Q., Lin, L., Zhuang, J., Pang, C., Xie, T., Liu, Y., 2012. The structural changes of the bagasse

- hemicelluloses during the cooking process involving active oxygen and solid alkali. *Carbohydrate Research* 359 (0), 65–69.
- Singh, P., Suman, A., Tiwari, P., Arya, N., Gaur, A., Shrivastava, A., 2008. Biological pretreatment of sugarcane trash for its conversion to fermentable sugars. *World Journal of Microbiology and Biotechnology* 24 (5), 667–673.
- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in biomass. *Laboratory Analytical Procedure*.
- Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology* 83 (1), 1–11.
- Taherzadeh, M., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. *International Journal of Molecular Sciences* 9 (9), 1621–1651.
- Viikari, L., Vehmaanperä, J., Koivula, A., 2012. Lignocellulosic ethanol: From science to industry. *Biomass and Bioenergy*.
- Wan, P., Zhai, D., Wang, Z., Yang, X., Tian, S., 2012. Ethanol Production from Nondetoxified Dilute-Acid Lignocellulosic Hydrolysate by Cocultures of *Saccharomyces cerevisiae* Y5 and *Pichia stipitis* CBS6054. *Biotechnology Research International* 2012.
- Wettstein, S., Alonso, D., Gürbüz, E., Dumesic, J., 2012. A roadmap for conversion of lignocellulosic biomass to chemicals and fuels. *Current Opinion in Chemical Engineering* 1 (3), 218 – 224.
- Wyman, C., Dale, B., Elander, R., Holtzapple, M., Ladisch, M., Lee, Y., 2005. Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology* 96 (18), 1959–1966.
- Yan, W., Acharjee, T., Coronella, C., Vásquez, V., 2009. Thermal pretreatment of lignocellulosic biomass. *Environmental Progress & Sustainable Energy* 28 (3), 435–440.
- Yang, B., Wyman, C., 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts and Biorefining* 2 (1), 26–40.
- Zhang, A., Liu, C., Sun, R., 2012a. Dissolution of cellulose in ionic liquids assisted with ethanol pretreatment. *Advanced Materials Research* 538, 2429–2433.
- Zhang, J., Ren, X., Chen, W., Bao, J., 2012b. Biological pretreatment of corn stover by solid state fermentation of *phanerochaete chrysosporium*. *Frontiers of Chemical Science and Engineering*, 1–6.
- Zhu, Z., Zhu, M., Wu, Z., 2012. Pretreatment of sugarcane bagasse with  $\text{NH}_4\text{OH-H}_2\text{O}_2$  and ionic liquid for efficient hydrolysis and bioethanol production. *Bioresource Technology* 119 (0), 199 – 207.

*E. Research paper III*  
*(Paper IV)*

---

*Effects of inhibitors formed during bagasse  
pretreatment on ethanol fermentation  
by Pichia stipitis CBS6054*

RAJIB BISWAS, H. UELLEND AHL AND B. K. AHRING

*(Intended for submission to Biomass & Bioenergy)*

# Effects of inhibitors formed during bagasse pretreatment on ethanol fermentation by *Pichia stipitis* CBS6054

Rajib Biswas<sup>a,b</sup>, H. Uellendahl<sup>a</sup>, B.K. Ahring<sup>a,b</sup>

<sup>a</sup>Section for Sustainable Biotechnology, Aalborg University Copenhagen, A. C. Meyers Vænge 15, 2450 Copenhagen SV, Denmark

<sup>b</sup>Center for Bioproducts and Bioenergy, Washington State University, 2710 University Drive, Richland, WA 99354-1671, USA (Email: bka@tricity.wsu.edu)

---

## Abstract

Sugarcane bagasse is a potential feedstock for cellulosic ethanol production, rich in glucan and xylan. The importance of utilizing all hydrolyzed sugar monomers into ethanol for improving process economics is self-evident. During processing of the hydrolysate, however, degradation products such as acetate, 5-hydroxymethylfurfural (HMF) and furfural are formed, which are known to inhibit microbial growth. In the current study, effects of the inhibitors in various concentration were investigated on wet exploded bagasse hydrolysates without detoxification using *Pichia stipitis* CBS6054, a native xylose utilizing yeast strain. The sugar utilization ratio and  $Y_{p/s}$  ranged from 88–100% and 0.33–0.41±0.02 g/g, respectively, in all the hydrolysates and controls tested. For the hydrolysate after wet explosion at 185 °C and 6 bar O<sub>2</sub>, composed of mixed sugars (glucose and xylose) and inhibitors such as acetate, HMF and furfural at concentrations of 3.2±0.1, 0.4 and 0.5 g/L, respectively, exhibited highest cell growth rate at 0.09 g/L/h. *Pichia stipitis* exhibited prolonged fermentation time on bagasse hydrolysate after wet explosion at 200 °C and 6 bar O<sub>2</sub> where the inhibitors concentration was further increased. Nonetheless, ethanol concentration was obtained up to 18.7±1.1 g/L resulting in a yield of 0.38±0.02 g/g after 82 h of fermentation.

**Keywords:** *Pichia stipitis*, cellulosic ethanol, bagasse, pretreatment, inhibitors, xylose fermentation.

---

## 1. Introduction

In recent years, ethanol production from renewable sources has received increased attention in a world of dwindling fossil fuels reserves along with the environmental concerns. Commercial production of bioethanol is mostly driven by starch- or sucrose-containing feedstocks such as corn, sugarcane, wheat by fermentation with *Saccharomyces cerevisiae* (Wheals et al., 1999). Non-food feedstocks, however, such as lignocellulosic materials including agricultural wastes such as bagasse hold significant potentials and have been identified as suitable feedstock sources for ethanol production (Lynd et al., 1991). Lignocellulose based ethanol process requires pretreatment as a first step followed by enzymatic hydrolysis of carbohydrates, that is cellulose and hemicellulose (Ahring et al., 1996; Margeot et al., 2009). Unlike the hydrolysis of starch- and sugar-based feedstock that results primarily in hexoses, lignocellulose is composed of cellulose and hemicellulose, resulting in both hexose (C6) and pentose (C5) sugars (Rubin,

November 5, 2012

2008). An efficient pretreatment strategy along with the fermentation of C6 and C5 sugars are the keys to bring cellulosic ethanol to commercial reality.

Sugarcane bagasse (SCB), the residual plant material of sugarcane, is one of the most abundant lignocellulosic feedstocks suitable for ethanol production (Cardona et al., 2010; Pandey et al., 2000). In addition, its on-site availability at sugarcane-based ethanol process plant is advantageous for large-scale processing. Currently the bagasse generated after sucrose extraction from sugarcane is incinerated to power the plant operation (Shi et al., 2012). SCB is primarily composed of cellulose (40-45%), hemicelluloses (30-35%) and lignin (20-30%) (Cardona et al., 2010). Cellulose is a D-glucose polymer while hemicellulose predominately consists of D-xylose, a five-carbon sugar (Jeffries et al., 2007; Skoog and Hahn-Hägerdal, 1990). An appropriate pretreatment is essential for efficient enzymatic saccharification as lignin hinders the process otherwise. Various pretreatment methods have shown the potential to disrupt the cell wall structure of SCB to facilitate the enzymatic hydrolysis of the polysaccharides (Cardona et al., 2010; Martín et al., 2007). Wet explosion is a thermochemical pretreatment method, where biomass is treated at high temperature and pressure. Typically an oxidizing agent such as elemental oxygen or H<sub>2</sub>O<sub>2</sub> is added to help disrupting the cell wall structure, solubilizing hemicellulose and lignin. The process is terminated by sudden pressure release to a subsequent flash tank (Ahring and Munck, 2006; Rana et al., 2012). In previous studies, the potential of wet explosion pretreatment of bagasse to facilitate saccharification at low enzyme dosage was demonstrated (Biswas et al., [Unpublished]). The oxidative pretreatment strategy was found to improve the cellulose conversion to glucose in the subsequent enzymatic hydrolysis, but also producing high xylose yields through solubilization of hemicellulose. However, during the processing of hydrolysate for subsequent microbial fermentation, degradation products such as acetate, 5-hydroxymethylfurfural (HMF), furfural are likely to be formed, which are known to inhibit the microbial growth and product yields (Bellido et al., 2011; Nigam, 2001a; Palmqvist and Hahn-Hägerdal, 2000).

The importance of utilizing all hydrolyzed sugar monomers into ethanol for improving process economics is self-evident. *Saccharomyces cerevisiae* is the most commonly used yeast for industrial ethanol fermentation, only capable of glucose fermentation. Some naturally occurring yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* are able to ferment both hexoses and pentoses to ethanol. Among the xylose fermenting yeasts, *Pichia stipitis* seems to be the most promising strain for industrial application due to its high ethanol



yield. In addition, this organism is able to ferment most of the sugars glucose, xylose, mannose, galactose and cellobiose (Agbogbo and Coward-Kelly, 2008). However, previous studies have shown arabinose is only utilized by *P. stipitis* for cell growth but not for ethanol production (Nigam, 2001b). Furthermore, it also has the natural ability to metabolize some of the sugar degradation compounds present in the hydrolysate after pretreatment (Almeida et al., 2008; Wan et al., 2012). The sensitivity of *Pichia stipitis* to inhibitors found in lignocellulose hydrolysate has been reported elsewhere (Bellido et al., 2011; Delgenes et al., 1996).

Inhibitory compounds, such as acetic acid, HMF and furfural are produced in different concentrations depending on the pretreatment severity and can inhibit the growth of yeast cell and thus lower the yield and productivity of ethanol fermentation. It was previously reported that prolonged incubation helps to acclimatize *Pichia stipitis* to these toxic compounds (Delgenes et al., 1996). In the present study, we investigated the effects of the different levels of inhibitors, that are generated during wet explosion pretreatment of SCB, on cell growth and ethanol yields by *P. stipitis* CBS6054 for both hexose and pentose sugars fermentation. We further compared the cell growth and yields using bagasse xylose hydrolysate containing only pentose sugar with inhibitors but in lower concentrations. The kinetics of cell growth in the hydrolysates compared to synthetic media were also assessed.

## **2. Materials and Methods**

### *2.1. Yeast strain and inoculum preparation*

*P. stipitis* CBS6054 was obtained from the American Type Culture Collection (ATCC) and was preserved at  $-80^{\circ}\text{C}$  in Bioproducts, Sciences and Engineering Laboratory (BSEL), Washington State University, USA. The organism was cultivated in a media previously described elsewhere (Agbogbo and Wenger, 2006, 2007). A mixture of yeast extract, urea, peptone and xylose (YUPX) in the respective proportions of 1.7, 2.27, 6.65 and 20.0 g/L was filter sterilized ( $0.22\ \mu\text{m}$ ) and used as source of nutrients. 250 mL sterilized Erlenmeyer baffled flasks were used and inoculation was done aseptically. The inoculated medium was incubated in a shake incubator at  $30^{\circ}\text{C}$  with agitation of 140 rpm for 48h. Microaerophilic conditions were maintained by using foam plugs on the Erlenmeyer flasks (Identi-Plugs<sup>®</sup>, Jaece Industries, Inc., NY). *P. stipitis* cells were harvested towards the end of the exponential growth phase by centrifugation at 6000 rpm for 10 mins. The harvested cells were washed twice and resuspended in sterilized distilled water in the desired cell concentration and served as inoculum.

## 2.2. Wet explosion pretreatment

Wet explosion pretreatment was performed at WSU pretreatment pilot plant for disrupting the lignocellulosic matrix and fractioning the lignin and hemicellulosic components as previously described (Rana et al., 2012). Sugarcane bagasse was added to the reactor as wet slurry with 16.0% dry matter concentration, containing 640 g of oven dry bagasse and 3343 g of tap water. The reactor was hermetically closed, 6 bar of O<sub>2</sub> was then purged into the reactor and the reactor was heated to the desired temperature. Reaction time was 10 minutes at the constant temperature and pressure. Three suitable pretreatment conditions were chosen based on preliminary results on enzymatic hydrolysis of wet exploded bagasse (Table 1). However, higher enzyme efficiency and recovery of both glucose and xylose obtained under condition B followed by condition C (Biswas et al., [Unpublished]), while condition A has been used as a control.

Table 1: Wet explosion pretreatment conditions applied on sugarcane bagasse with a treatment time of 10 minutes.

Pretreatment	Temperature, °C	Pressure, bar		pH		Dry matter, %	
		Initial (O <sub>2</sub> )	Final	Initial	Final	Initial	Final
A	170	6	17	5.85	3.12	16.0	15.5
B	185	6	19	5.85	3.05	16.0	16.2
C	200	6	22	5.85	2.93	16.0	14.0

## 2.3. Preparation of hydrolysates from wet exploded bagasse

### 2.3.1. Hydrolysate with mixed sugars after enzymatic hydrolysis

After pretreatments under condition B and C (Table 1), enzymatic hydrolysis was carried out on the whole wet exploded material without any solid-liquid separation. For saccharification, a mixture of the two commercial enzymes Cellic<sup>®</sup>CTec2 and Cellic<sup>®</sup>HTec2 (Novozymes, USA) were used in a ratio of 85:15 (% v/v), respectively, with the enzyme loading of 12.4 enzyme protein (EP)/g cellulose at 10.1±0.1% dry matter. The enzyme protein (EP) content of Cellic<sup>®</sup>CTec2 and Cellic<sup>®</sup>HTec2 determined prior to enzymatic hydrolysis were 279 ± 8 and 251 ± 12 mg EP/mL, respectively. Hydrolysates were always filter sterilized (0.2 µm, millipore, USA) prior to inoculation.

### 2.3.2. Xylose hydrolysate after SSF

Xylose fraction of the hydrolysate was obtained after simultaneous saccharification and fermentation (SSF) of wet exploded bagasse at condition A (Table 1) using *Saccharomyces cerevisiae* for 162 hours. The liquid fraction of the hydrolysate contains only sugar xylose was separated by filtration. While SSF was completed, ethanol produced during SSF was removed by vacuum distillation prior to the separation.

### 2.4. Shake flask fermentation

Shake flask fermentation was conducted in duplicates with the hydrolysates (Table 2) under same conditions for temperature and agitation as previously described (Section 2.1). Erlenmeyer baffled flasks (50 mL) containing 30 mL of the fermentation medium were inoculated with 1 mL of cell suspensions to reach a cell concentration of 1 g/L. In addition, 1 mL of nutrient solution was supplied to each flask. Nutrient solution (50 mL) was prepared by dissolving 4.25 g of yeast extract, 5.675 g of urea and 16.4 g of peptone in water. All fermentation flasks were supplied with sufficient carbon sources (i.e., hydrolysate or commercial sugar) and nutrients to produce equivalent amount of cell mass and to exhibit similar growth rates under the favorable conditions ensured. The flasks were incubated for 106 hours except for hydrolysate  $C_{GX}$  which was incubated for 174 hours. 2 mL of sample was withdrawn after 0, 6, 12, 24, 36, 48, 58, 82, 106 and 174 hours (in case of hydrolysate  $C_{GX}$ ) for analysis of sugar and inhibitor concentration, cell concentration and pH.

Table 2: Composition (g/L) of the substrates used for fermentation by *Pichia stipitis*.

Substrate	Initial sugar concentration		Inhibitor concentration		
	Glucose	Xylose	Acetic acid	HMF	Furfural
$A_X$ [ <sup>a</sup> ]	0.0	14.7±0.0	1.0±0.0	1.2±0.0	0.4±0.0
$B_{GX}$ [ <sup>b</sup> ]	17.3±0.5	9.6±0.2	3.2±0.1	0.4±0.0	0.5±0.0
$C_{GX}$ [ <sup>c</sup> ]	42.8±0.8	6.3±0.0	6.9±0.1	1.2±0.0	0.8±0.0
$S_{GX}$ [ <sup>d</sup> ]	6.1±0.0	15.2±0.0	0.0	0.0	0.0
$S_G$ [ <sup>d</sup> ]	27.2±0.5	0.0	0.0	0.0	0.0
$S_X$ [ <sup>d</sup> ]	0.0	25.6±0.4	0.0	0.0	0.0

<sup>a</sup>Hydrolysate after pretreatment at condition A and SSF using *Saccharomyces cerevisiae*.

<sup>b</sup>Hydrolysate after pretreatment at condition B and enzymatic hydrolysis.

<sup>c</sup>Hydrolysate after pretreatment at condition C and enzymatic hydrolysis.

<sup>d</sup>Respective commercial sugar (granular powder) was used as control substrate, Fisher Chemical, USA.

## 2.5. Analytical methods

Cell concentrations were determined by optical density (OD) measurement of the cells using spectrophotometer (Jenway 6405 UV/Visible, NJ, USA) system at 600 nm (1 OD = 0.17 g/L of dry cells). Glucose, xylose, arabinose, acetic acid, ethanol, HMF and furfural were quantified by HPLC on an Aminex HPX-87H column (Bio-Rad, Hercules, USA) at 60 °C with 4 mM H<sub>2</sub>SO<sub>4</sub> as an eluent with a flow rate of 0.6 mL/min. HPLC was equipped with refractive index and UV visible detector. All samples were filtered through a 0.45 µm PTFE membrane (Acrodisc® Syringe Filters, 13mm, Pall® Life Sciences, USA) prior to HPLC analysis. The pH was monitored using InLab® Micro combination pH electrode (precision ±0.001 pH).

## 3. Results and discussion

### 3.1. Effects of inhibitors on sugar utilization and ethanol yields

The main parameter of the fermentation by *Pichia stipitis* CBS6054 on the different hydrolysates and control media are displayed in table 3. Sugar utilization, ethanol yields, inhibitor concentration, pH and growth kinetics of hydrolysates B<sub>GX</sub> and C<sub>GX</sub> are presented in figure 1.

The sugar utilization ratio and Y<sub>p/s</sub> ranged from 88–100% and 0.33–0.41±0.02 g/g, respectively, in all the hydrolysates and controls tested. The ethanol yields (Y<sub>p/s</sub>) of hydrolysate A<sub>X</sub>, B<sub>GX</sub> and C<sub>GX</sub> were 0.41±0.02, 0.39±0.02 and 0.38±0.02 g/g, respectively. Ethanol yields were higher when using hydrolysates after pretreatment than control substrates, i.e., commercial sugars (Table 3). The yields are in agreement with the results found in corn stover hemicellulose hydrolysate with similar inhibitors concentration using *Pichia stipitis* CBS6054 (Agbogbo and Wenger, 2007). Our results are comparable to those observed with adapted *Pichia stipitis* strains (Nigam, 2001a,b).

The utilization of glucose was more rapid than of xylose in the different hydrolysates. The similar observation in assimilation of sugars was reported elsewhere (Agbogbo and Wenger, 2007; Bellido et al., 2011; Nigam, 2001a). In the presence of both glucose and xylose (B<sub>GX</sub>, C<sub>GX</sub>), conversion of glucose started prior to xylose conversion. In a mixed substrate fermentation, significant xylose utilization is initiated by *Pichia stipitis* once glucose concentration in the medium is below 20 g/L (Agbogbo et al., 2006).

Both glucose and xylose were converted for the hydrolysates B<sub>GX</sub> and C<sub>GX</sub>, containing inhibitors in the higher concentrations after the pretreatment and enzymatic hydrolysis of SCB

Table 3: Summaries of fermentation results at highest ethanol concentration time points using *Pichia stipitis*.

Substrate	Fermentation time, (f)h	Ethanol, g/L	Sugar utilized, %	$Y_{p/s}^a$ , g/g	Cell growth rate, g/L/h	Cell mass, g/L at (f)h	Cell mass, g/L at 106 h	pH at (f)h
A <sub>X</sub>	58	6.1±0.0	100±0.0	0.41±0.02	0.05	2.81±0.02	3.34±0.02	5.5±0.0
B <sub>GX</sub>	36	10.4±0.2	100±0.0	0.39±0.02	0.09	3.31±0.00	4.02±0.02	6.7±0.0
C <sub>GX</sub>	82	18.7±1.1	88±0.0	0.38±0.02	0.04	3.16±0.00	3.52±0.09	6.2±0.0
S <sub>GX</sub>	76	8.2±0.0	100±0.0	0.39±0.00	0.03	2.48±0.00	2.69±0.00	6.6±0.0
S <sub>G</sub>	36	10.1±0.1	99±0.8	0.37±0.00	0.06	2.33±0.05	2.72±0.04	6.0±0.3
S <sub>X</sub>	82	8.5±0.2	100±0.0	0.33±0.01	0.03	2.81±0.03	2.94±0.05	5.3±0.1

<sup>a</sup> $Y_{p/s}$  = ethanol yield coefficient, was calculated as the grams of ethanol produced per grams of sugar converted.

(Figure 1). Our results compare favorably with previous reports on fermentation of sugar cane bagasse hydrolysate by Rudolf et al. (2008). The fermentation of xylose alone after pretreatment at condition A (170 °C, 6 bar O<sub>2</sub>) and SSF took 58 h (Table 3), which is longer than that of mixed sugars in the hydrolysate B<sub>GX</sub> after pretreatment condition B (185 °C, 6 bar O<sub>2</sub>), which took 36 h (Figure 1). Fermentation of enzymatic hydrolysate C<sub>GX</sub> after pretreatment at condition C (200 °C, 6 bar O<sub>2</sub>) resulted in prolonged fermentation time of 82 h with initial lag phase of 12 h. The delay in sugar conversion is likely due to the presence of inhibitors such as acetate, HMF and furfural at the concentrations of 6.9±0.1, 1.2 and 0.8 g/L, respectively. Nonetheless, ethanol concentration (18.7±1.1 g/L) observed was noteworthy after 82 h of incubation and after adaptation to the inhibitors, the fermentation was completed with an ethanol yield of 0.38±0.02 g/g. Although the utilization of sugars was limited to 88% (Figure 1), sugar conversion was increased up to 95% after 174 h of fermentation. In contrast, Bellido et al. (2011) reported no utilization of xylose in 168 h using *P. stipitis* DSM3651 in the experiment on filtered enzymic hydrolysate of steam exploded wheat straw using the whole slurry with acetate, HMF and furfural concentration at 1.52, 0.05 and 0.14 g/L, respectively.

### 3.2. Effects of inhibitors on cell growth

When comparing the growth kinetics of *Pichia stipitis* CBS6054 in figure 1, the initial cell concentration of 1 g/L increased for all hydrolysates and grew to various final cell concentrations on the different hydrolysate medium. Highest amount of cell mass (g/L) produced in mixed sugars hydrolysate B<sub>GX</sub> after 106 h of incubation was 4.02±0.02, while 3.34±0.02 and 3.52±0.09 in hydrolysate A<sub>X</sub> and hydrolysate C<sub>GX</sub>, respectively (Table 3).

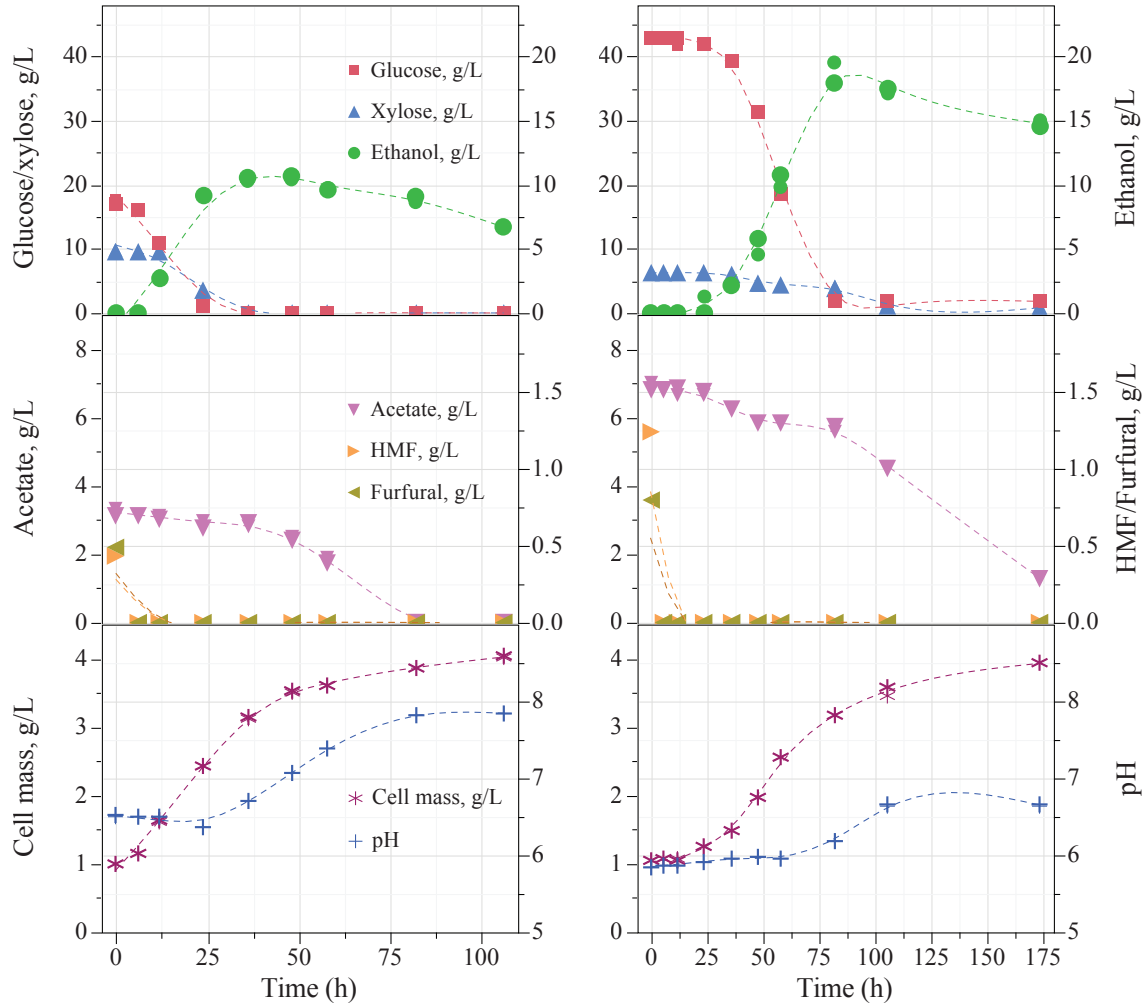


Figure 1: Sugar conversion and ethanol production (up); conversion of inhibitors (middle); and cell growth and pH (bottom) in batch fermentation of hydrolysates with *Pichia stipitis* strain CBS6054; Hydrolysate B<sub>GX</sub> (left), after enzymatic hydrolysis of wet exploded bagasse under condition at 185 °C with 6 bar O<sub>2</sub>; Hydrolysate C<sub>GX</sub> (right), after enzymatic hydrolysis of wet exploded bagasse under condition at 200 °C with 6 bar O<sub>2</sub>.

Cell growth rate and mass production were higher in all hydrolysates than found in synthetic medium (S<sub>GX</sub>, S<sub>G</sub> and S<sub>X</sub>). Exponential growth was observed for hydrolysate A<sub>X</sub> and B<sub>GX</sub> (Figure 1) during the initial 48 h without any noticeable lag phase. Cell mass in hydrolysate A<sub>X</sub> and B<sub>GX</sub> after 48 h were measured to 2.81 and 3.52 g/L, respectively. The favorable growth condition for cell mass production is likely due to the mixed sugars, where glucose is converted more readily than xylose. On the other hand, no cell growth was observed in hydrolysate C<sub>GX</sub> within the first 12 h (Figure 1). This lag phase is possibly due to a higher concentration of inhibitor in hydrolysate C<sub>GX</sub> such as acetate (6.9±0.1 g/L), HMF (1.2 g/L) and furfural (0.8 g/L). Similar observation was also reported by Agbogbo and Wenger (2007) and Sreenath and Jeffries (2000). However, after adaptation to the hydrolysate C<sub>GX</sub> within 12 h, exponential growth was

observed. The performance was significantly improved shortly after 12 h of incubation. This lag phase can be overcome in a continuous process using initial high cell density and also by recycling the cells adapted to the inhibitors (Bellido et al., 2011).

The highest cell growth rate of 0.09 g/L/h was found in hydrolysate B<sub>GX</sub> followed by 0.06 g/L/h in synthetic media S<sub>G</sub> (Table 3). It was observed that the fermentation performance was not disturbed rather improved in the hydrolysates containing lower level of inhibitors (as given in Table 2) compared to the commercial sugar mediums (controls). Acetic acid concentrations in the hydrolysates A<sub>X</sub>, B<sub>GX</sub> and C<sub>GX</sub> were 1.0±0.0, 3.2±0.1 and 6.9±0.1 g/L, respectively (Table 2). Acetic acid is released from the esterified form of arabinoxylans during the processing of lignocellulose hydrolysate. The cleavage of the acetyl group occurs when lignocellulose undergoes high temperature, oxidation treatment and even in enzymatic hydrolysis process we see a liberation acetic acid. Previous studies showed the yeast cell growth is inhibited at an acetic acid concentration of about 2-5 g/L (Bellido et al., 2011; Nigam, 2001a).

Taking into consideration that no detoxification was performed except the adjustment of pH with NaOH to 6.0±0.5, it was found that the fermentation was only inhibited in bagasse hydrolysate C<sub>GX</sub> after pretreatment at condition C (200 °C, 6 bar O<sub>2</sub>). Acetic acid was converted in all fermentation experiments especially with hydrolysate B<sub>GX</sub> and C<sub>GX</sub> resulting an increase in pH (Agbogbo and Wenger, 2007) (Figure 1). After 82 h of fermentation, 100% acetic acid was metabolized in hydrolysate B<sub>GX</sub>. Hence, for the hydrolysate C<sub>GX</sub>, it took 174 h to bring the acetic acid concentration to 1.3 g/L from 6.9±0.1 g/L. A similar observation on acetic acid conversion by *Pichia stipitis* was also reported by Agbogbo and Wenger (2007) during fermentation of corn stover hydrolysate. The product from acetic acid metabolism by *Pichia stipitis* CBS6054 is unknown.

HMF and furfural are produced during the processing of hydrolysate, by degradation of hexose and pentose sugars, respectively. Apparently, the tested concentration levels of HMF and furfural were not affecting the fermentation and growth of *Pichia stipitis* CBS6054. Yeasts including *Pichia stipitis* can metabolize furfural to furfuryl alcohol and the enzyme NADH-dependent yeast alcohol dehydrogenase (ADH) is responsible for the reduction (Huang et al., 2009). In the present investigation, HMF and furfural were completely metabolized by the strain before significant utilization of sugars started. This was also reported by Almeida et al. (2008) and Wan et al. (2012). This indicates that *Pichia stipitis* CBS6054 is readily capable of converting HMF and furfural in the tested lignocellulose hydrolysate from sugarcane bagasse.

#### 4. Conclusion

To realize the industrial ethanol production from hydrolysis of pretreated lignocellulose, it is essential to obtain strains capable of converting the major sugars, that is glucose and xylose, as well as limiting the inhibitory effects of the degradation products in the hydrolysate from the pretreatment. This current work demonstrates that the native strain *Pichia stipitis* CBS6054 is suitable for ethanol fermentation of both glucose and xylose present in hydrolysate of wet exploded bagasse without the need for detoxification, achieving substantial ethanol yields. The ethanol yield from xylose of the hydrolysate after pretreatment at 170 °C with 6 bar O<sub>2</sub> and SSF was 0.41±0.02 g/g while a yield of 0.39±0.02 g/g was achieved for the fermentation of glucose and xylose in hydrolysate after pretreatment at 185 °C with 6 bar O<sub>2</sub> and enzymatic hydrolysis of wet exploded bagasse. Cell growth was highest (0.09 g/L/h) in hydrolysate containing mixed sugars and inhibitors such as acetate, HMF and furfural concentration at 3.2±0.1, 0.4 and 0.5 g/L, respectively, meaning that the processing of bagasse hydrolysate under this condition (185 °C with 6 bar O<sub>2</sub>) will not inhibit the growth of *Pichia stipitis*. Although *Pichia stipitis* exhibited prolonged fermentation time on the hydrolysate C with higher inhibitor concentration, ethanol concentration up to 18.7±1.1 g/L was obtained yielding 0.38±0.02 g/g after 82 h. The fermentation time is expected to be reduced by increasing the initial cell density and recycling the adapted cells in a continuous process.

#### Acknowledgment

The authors would like to express gratitude to the Strategic Research Council for support to the Biorefinery project which has funded this work. We further thank Jens Iversen for technical input for the fermentation.



## References

- Agbogbo, F., Coward-Kelly, G., 2008. Cellulosic ethanol production using the naturally occurring xylose-fermenting yeast, *Pichia stipitis*. *Biotechnology Letters* 30 (9), 1515–1524.
- Agbogbo, F., Wenger, K., 2006. Effect of pretreatment chemicals on xylose fermentation by *Pichia stipitis*. *Biotechnology Letters* 28 (24), 2065–2069.
- Agbogbo, F., Wenger, K., 2007. Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*. *Journal of Industrial Microbiology & Biotechnology* 34 (11), 723–727.
- Agbogbo, F. K., Coward-Kelly, G., Torry-Smith, M., Wenger, K. S., 2006. Fermentation of glucose/xylose mixtures using *Pichia stipitis*. *Process Biochemistry* 41 (11), 2333 – 2336.
- Ahring, B., Jensen, K., Nielsen, P., Bjerre, A., Schmidt, A., 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. *Bioresource Technology* 58 (2), 107–113.
- Ahring, B., Munck, J., Mar. 30 2006. Method for treating biomass and organic waste with the purpose of generating desired biologically based products. WO 2006/032282 A1.
- Almeida, J., Modig, T., Röder, A., Lidén, G., Gorwa-Grauslund, M., et al., 2008. *Pichia stipitis* xylose reductase helps detoxifying lignocellulosic hydrolysate by reducing 5-hydroxymethyl-furfural (HMF). *Biotechnol Biofuels* 1 (1), 12.
- Bellido, C., Bolado, S., Coca, M., Lucas, S., González-Benito, G., García-Cubero, M. T., 2011. Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*. *Bioresource Technology* 102 (23), 10868–10874.
- Biswas, R., Uellendahl, H., Ahring, B., [Unpublished]. Wet explosion pretreatment of sugarcane bagasse for enhanced enzymatic hydrolysis. In preparation.
- Cardona, C., Quintero, J., Paz, I., 2010. Production of bioethanol from sugarcane bagasse: status and perspectives. *Bioresource Technology* 101 (13), 4754–4766.
- Delgenes, J., Moletta, R., Navarro, J., 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. *Enzyme and Microbial Technology* 19 (3), 220–225.
- Huang, C., Lin, T., Guo, G., Hwang, W., 2009. Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of *pichia stipitis*. *Bioresource Technology* 100 (17), 3914–3920.
- Jeffries, T., Grigoriev, I., Grimwood, J., Laplaza, J., Aerts, A., Salamov, A., Schmutz, J., Lindquist, E., Dehal, P., Shapiro, H., et al., 2007. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nature Biotechnology* 25 (3), 319–326.
- Lynd, L., Cushman, J., Nichols, R., Wyman, C., 1991. Fuel ethanol from cellulosic biomass. *Science* 251 (4999), 1318–1323.
- Margeot, A., Hahn-Hagerdal, B., Edlund, M., Slade, R., Monot, F., 2009. New improvements for lignocellulosic ethanol. *Current opinion in Biotechnology* 20 (3), 372–380.
- Martín, C., Klinke, H., Thomsen, A., 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. *Enzyme and Microbial Technology* 40 (3), 426–432.

- Nigam, J., 2001a. Ethanol production from hardwood spent sulfite liquor using an adapted strain of *Pichia stipitis*. *Journal of Industrial Microbiology & Biotechnology* 26 (3), 145–150.
- Nigam, J., 2001b. Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. *Journal of Biotechnology* 87 (1), 17–27.
- Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresource Technology* 74 (1), 17–24.
- Pandey, A., Soccol, C., Nigam, P., Soccol, V., 2000. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresource Technology* 74 (1), 69–80.
- Rana, D., Rana, V., Ahring, B. K., 2012. Producing high sugar concentrations from loblolly pine using wet explosion pretreatment. *Bioresource Technology* 121 (0), 61–67.
- Rubin, E., 2008. Genomics of cellulosic biofuels. *Nature* 454 (7206), 841–845.
- Rudolf, A., Baudel, H., Zacchi, G., Hahn-Hägerdal, B., Lidén, G., 2008. Simultaneous saccharification and fermentation of steam-pretreated bagasse using *Saccharomyces cerevisiae* TMB3400 and *Pichia stipitis* CBS6054. *Biotechnology and Bioengineering* 99 (4), 783–790.
- Shi, J., Yang, Q., Lin, L., Zhuang, J., Pang, C., Xie, T., Liu, Y., 2012. The structural changes of the bagasse hemicelluloses during the cooking process involving active oxygen and solid alkali. *Carbohydrate Research* 359 (0), 65–69.
- Skoog, K., Hahn-Hägerdal, B., 1990. Effect of oxygenation on xylose fermentation by *Pichia stipitis*. *Applied and Environmental Microbiology* 56 (11), 3389–3394.
- Sreenath, H., Jeffries, T., 2000. Production of ethanol from wood hydrolyzate by yeasts. *Bioresource Technology* 72 (3), 253–260.
- Wan, P., Zhai, D., Wang, Z., Yang, X., Tian, S., 2012. Ethanol Production from Nondetoxified Dilute-Acid Lignocellulosic Hydrolysate by Cocultures of *Saccharomyces cerevisiae* Y5 and *Pichia stipitis* CBS6054. *Biotechnology Research International* 2012.
- Wheals, A., Basso, L., Alves, D., Amorim, H., 1999. Fuel ethanol after 25 years. *Trends in Biotechnology* 17 (12), 482–487.

## Concluding remarks

If lignocellulosic feedstock is to play a dominant role in future biorefineries an efficient pretreatment method like wet explosion will be the key to tapping into the potential of this abundant renewable resource. The advantages of using wet explosion (WEx) pretreatment in future biorefineries include higher enzymatic efficiency in hydrolysis of the polysaccharides to obtain an intermediate platform for subsequent bioconversion processes. Another remarkable advantage is that the estimated costs of lignocellulose processing can be significantly reduced by introducing WEx pretreatment in biorefineries, since no recovery of the chemicals/catalysts will be necessary as well as reducing the cost of enzymes for a successful hydrolysis using high solid materials. Furthermore, WEx pretreatment offers flexibility in feedstock selection, suitable for industrial implementation. In addition, during processing of lignocellulose, WEx pretreatment creates only low/tolerable amount of degradation products for subsequent microbial processes. In this study investigations were limited to yeast fermentation and anaerobic digestion for bioethanol and biogas production, respectively. The adjustment of the process parameters for the WEx pretreatment is essential for each biomass feed and the subsequent microbial processes in order to maximize the yields and to minimize the effects of the degradation products, if any.

A new concept for increasing methane yield of manure based biogas plant has been extensively tested as part of this study. WEx pretreatment of separated solids from the effluent of a commercial biogas digester contained digested manure fibers fraction, before reintroducing the solids into the digester for further digestion has been evaluated. Results in batch and reactor experiments showed the methane yield of the fiber fraction can be significantly improved. Optimal conditions were found to be a temperature of 180 °C without addition of any oxidizing agents with a treatment time of 10 minutes. Under the conditions, CH<sub>4</sub> yield has increased by 136% as compared with the untreated digested fibers. The continuous feeding of WEx-treated fibers in co-digestion with filtered manure revealed on average a 75% higher total yield with no significant signs of inhibition after a short adaptation phase. Economy assessment suggested that the methane yield per ton of feed could be increased from 23 to 28 m<sup>3</sup> by the recirculation and to 33 m<sup>3</sup> when the biodegradability of the fiber fraction is enhanced from 40 to 75%. The proof of this investigation in large scale will be followed by demo-scale testing of the concept at Biokrafts biogas plant on Bornholm, Denmark.

This dissertation investigated pilot-scale performance of WEx pretreatment of sugarcane bagasse for improving the enzymatic hydrolysis of the potential feedstock into monomeric compounds as intermediate platform for production of fuels, chemicals and value added products. The preferable conditions for wet explosion pretreatment of sugarcane bagasse were 185 °C with 6 bar O<sub>2</sub> pressure for 10 minutes. Under this pretreatment condition, solubilization of hemicellulose and lignin enriched the glucan content (59.2%, dry weight) in pretreated cake samples. Furthermore, the enzyme loading of 22.0 mg enzyme protein (EP)/g cellulose was efficient in glucan hydrolysis of washed cake sample, giving a glucose recovery up to 573.9±4.5 g/Kg TS with a glucose yield of 87.4±0.7% of the theoretical maximum value. Besides, total sugar monomers were found (glucose, xylose and arabinose, 34.0 g/L) after dilute acid hydrolysis of the liquid fraction under this condition. Although, the wet exploded bagasse contained degradation products in minor quantities under this condition (185 °C, 6 bar O<sub>2</sub>, 10 minutes), the effects of the inhibitors were further investigated using *Pichia stipitis* CBS6054 on ethanol fermentation of both glucose and xylose and on cell growth without any detoxification and compared with other samples. An ethanol yield of 0.39±0.02 g/g was achieved for the fermentation of glucose and xylose in hydrolysate after pretreatment at 185 °C with 6 bar O<sub>2</sub> and enzymatic hydrolysis of wet exploded bagasse. Moreover, cell growth was highest (0.09 g/L/h) in the hydrolysate containing mixed sugars and inhibitors such as acetate, HMF and furfural concentration at 3.2±0.1, 0.4 and 0.5 g/L, respectively.

This dissertation demonstrates the opportunities of lignocellulosic biorefineries using wet explosion pretreatment as a key method for biomass processing. The findings from both lab-scale and pilot-scale experimental works need to be testified in demo-scale and should lead to commercial implementation. Furthermore, focus on value added products and efforts to develop new biological routes are of paramount importance to make the process attractive for commercialization. In addition, investigation should not be limited to bench-scale but the large-scale demonstration of the features of lignocellulosic biorefineries will help to improve the understanding and to attract the attention of potential investors.